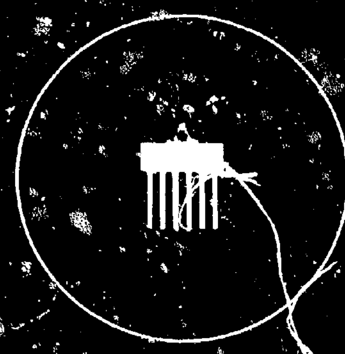
FOOD POISONING MICROORGANISMS

A STUDY OF CHARACTERISTICS
AND METHODS OF DETECTION
WITH PARTICULAR EMPHASIS
ON CLOSTRIDIUM PERFRINGENS



ACCOMPLISHED IN CONNECTION WITH
A SECRETARY OF THE ARMY'S
RESEARCH AND STUDY FELLOWSHIP

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by

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CONTENTS

CONTENTS (Continued)

CONTENTS (Continued)

CONTENTS (Continued)

TABLES

PREFACE

Food poisoning caused by Clostridium perfringens and efforts being made to detect and prevent it is the keynote of this Fellowship study. Since it is meant to come to the attention of administrators, some of whom may not be trained in the biological and physical sciences, I prepared as much of the report as possible in terms that, I trust, are understandable to the non-scientist official as well as to the food hygienist.

To avoid interruptions in the concepts presented, I have cited literature somewhat less formally than is conventional with scientific reports. The trend apparently is in this direction. Nevertheless, none of the references that I have drawn upon is lost sight of, for all are listed in the section devoted to this purpose. Carl Lamanna, in his superb text on basic bacteriology, ignores all citations to references while he is discussing a topic. Yet he includes a list of references, apropos of his subject, at the end of his treatise--where it does not interfere with smooth reading. The result is a highly communicative book. In this sense, I hope that my report emulates his text and communicates some insight into the role of C perfringens in food poisoning.

As an overall picture, a resume appears prior to the report per se. The foreword gives a preview of some current thoughts on C perfringens as a food poisoning agent. It also includes suggestions on important preventive measures. In the pages preceding the five basic parts of the report, background material is included justifying this study despite contrary opinions tending to minimize the problem. Each portion of the report, except part 5, is preceded by a synopsis of its contents, and is concluded with recommendations based on the observations made. These recommendations are recapitulated at the end of the study.

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ACKNOWLEDGEMENT AND THANKS TO ALL PARTICIPANTS

An expression of appreciation and high regard is due a number of persons for the opportunity afforded me and the assistance I received in connection with this Secretary of the Army Research and Study Fellowship.

Lieutenant Colonel Harvey L. Rubin, USA, who was Chief of the Defense Subsistence Supply Center's Laboratory when this Fellowship was awarded to me is recognized as its proponent. As my supervisor at that time, Dr. Rubin encouragingly judged my qualifications to be adequate and urged me to seek this Fellowship. He gave me material assistance in presenting the problem, and personally justified to evaluating officials the usefulness and need for undertaking this research study. In a similar vein I value the enlightened policy and foresight evident in the Fellowship program and the support and cooperation of the higher echelon officials at that time. These were Colonel Richard L. Lewis, Director of Technical Operations, Major General Hugh Mackintosh, Executive Director of the Defense Subsistence Supply Center, and the Honorable Elvis J. Stahr, Jr., Secretary of the Army. Likewise, I express my heartfelt gratitude for their continued efforts in my behalf during the course of this Fellowship, to the successors of these four men; namely, Lieutenant Colonel Thomas G. Murnane, Colonel John W. McAlhany, Major General Thomas B. Evans, and the Honorable Cyrus R. Vance. Without hiring a replacement, Dr. Murnane, Colonel McAlhany and Mr. Charles H. Coleman, the Laboratory's Chief Chemist, carried out my duties and responsibilities in addition to their own work during my absence.

Dr. Ralph H. Weaver, Professor of Bacteriology, is highly thought of for his talented contributions to the research and study effort associated with this Fellowship that was accomplished in his Department of Microbiology at the University of Kentucky. In the same manner Dr. Maurice Ingram, Deputy Director, and Dr. Ella M. Barnes, Principal Scientific Officer are praised for their inspiring direction and supervision of the investigation that was undertaken in the interest of this Fellowship project in their Low Temperature Research Station at the University of Cambridge in England. Dr. Betty C. Hobbs, Director, is commended for her gifted sponsorship of the study conducted on methods in her Food Hygiene Laboratory of the Ministry of Health in London.

The persons named in the section of this Fellowship report concerned with visitation of establishments are remembered for their cordial invitations to visit the activity they represent, and for the useful information they so generously provided. Finally, the members of the Fellowship Committee of the Army's Deputy Chief of Staff for Personnel, in particular Mr. S. Carlisle

Botts and Mrs. Josephine Wolfe, with whom I had frequent direct contact, are worthy of special mention for their apt administration and handling of the numerous intricate problems stemming from the implementation and execution of this Fellowship.

COMMENT ON PERSONAL EXPERIENCES

Notable advances in the sciences have been made in recent years both at home and abroad. The necessity for keeping abreast of, and enhancing scientific progress is fully realized in military channels. In consequence, the Secretary of the Army's Research and Study Fellowship program has undertaken the twofold responsibility of furthering research of major interest to military planning agencies, and of improving the knowledge and skills of its cadre of scientists. Our Department of Defense is lauded for this exemplary policy of sending its Fellowship recipients to institutions of learning that they have found possess special research talents and facilities. My dual choice of the University of Kentucky and the University of Cambridge was based on the more distinct interest of these two institutions in sponsoring the program I had proposed than twenty-eight others whom I had contacted.

It was a revelation to be a student at the University of Kentucky. Its charming campus and energetic student body restored me to the youthful college environment of yesteryear. This is seldom the privilege of a recipient to whom a Fellowship is awarded at the maximum age permitted. The high caliber of the faculty and staff at the University of Kentucky had a very constructive impact on my efforts there. Its physical plant and laboratory facilities are of the highest order. Its new medical library is of superb characteristics and dimensions. A pristine freshness pervades the colorfully bound journals, card catalogues, books, and reference papers. Even rare old volumes appear to have an up-to-dateness about them. The myriads of scientific journals and volumes it contains are more easily found, and more readily accessible to the user there than in any other library one can mention. So long as references, no matter how precious, are not needed by anyone else, the borrower may keep them as long as he wishes. The furniture, air conditioning, lighting, and accommodations provide an inspiring and exceptionally effective working environment. The new University of Kentucky library probably excels any outstanding technical library that was activated before it had been constructed. Certainly, it is the equal of any distinguished science library one could hope to find anywhere.

The people of Kentucky are as enchanting, as witty, and as intelligent as any one is likely to meet in the most sophisticated circles. It is unfortunate that an unflattering impression has been created of them in the contemptuous radio programs and music of Renfro Valley.

England is a much more attractive country than I expected to see. The pleasantly cool, moist climate is conducive to the cultivation of beautiful flowers. Prompted by such famed garths as those in Edinburgh, at Kew, and

Wye, outdoor living rooms of trim flower gardens are in blossom from early spring to late fall in all communities on the islands. This generates an aura of neatness and tidiness everywhere.

The visitor is welcome in England and left to go about his business unmolested. Unlike in other European or Latin American countries, he is spared the usual gamut of souvenir vendors, shoe shine boys, and gift seekers. He can feel secure and protected in Britain. Overly generous tipping is frowned upon, and is not expected. One shilling, equivalent to fourteen cents in the United States, is cheerfully received as recompense to check your coat, or carry your suitcase, or as a gratuity for a taxi fare or a luncheon check. British people are a peaceful, law abiding and decent folk. Gentleness, compromise, concession, and temperance permeate the land. Americans appreciate their fine manners, conservative thinking, compassion, lack of greed, and noticeable loyalty to their Government. They demonstrate this loyalty by kind words for their Queen, and in other ways, but most evidently by sounding their national anthem and standing reverently at every theater performance and other public affairs.

The outdoor climate is not as inhospitable as is depicted in common talk. Temperatures are not expected to reach extremes in summer or winter. This is evident in outdoor installation of water pipes in the optimistic anticipation that the winter will not be so severe as to freeze and burst them. A cool summer is always provided for, since beds are made up with heavy quilts and comforters even in July and August. Beverages are never refrigerated but are served at the temperature prevailing in the cellar, and 75 per cent of the homes have no ice boxes.

Strangely enough, during the greater part of the year the temperature indoors is practically the same as outdoors. In cold weather only a meager amount of heating is provided for because fuel is three times as costly in Britain as it is in the United States, while salaries are appreciably lower. An indoor temperature of 60 F is quite acceptable, but is often unattainable. For example, a large old home may have five different kinds of heat--fireplace, portable oil burners, gas jets, electric coils, and hot water radiators--all operating simultaneously for many hours. Yet enough heat will not be generated to reach 60 F when it is 35 F outdoors. This is due to the fact that the heating units have very small, toy-like capacities; there is no insulation in the construction; and ceilings are high. Britons accommodate themselves to these low temperature conditions by wearing heavy underwear, waistcoats, and sweaters. Throughout the year plates are heated before food is served on them. In winter, beds are preheated with a warming device such as a hot water bottle. Americans in Britain warm themselves locally by focussing divers heat sources directly on themselves at close proximity. An extreme

example of the Britisher's tolerance of a cold way of life is the use of water, drawn the night before in the wash basin for morning toilletry. After a frosty winter night the water may still be used unheated even if it is necessary to remove a sheet of ice formed on the surface.

Incidental to travel in connection with official business, my wife and I experienced a generous sample of life in English and Scottish homes, thanks to the hospitality of an alumnus of my alma mater, and new friends made overseas. John Page, resident of Solihull, England, had utilized a Fellowship at Illinois Institute of Technology, which had been awarded to him by his employer, an electric power company in nearby Birmingham. This happy coincidence led to a close friendship and three rewarding weekends spent with his lovely family in their home; and visits to the surrounding country immortalized by William Shakespeare. A similar opportunity was afforded to us by Dr. and Mrs. John G. Davis in their home in Reading, England. Dr. Davis was furthermore instrumental in arranging an all-day visit to the National Institute for Research in Dairying at the University of Reading, and a like visit to his consulting and research facilities in London. Besides the intimate experiences as resident guests in these two English homes, we had the pleasure of attending a number of social functions in other homes in England and Scotland.

My fellowship itinerary afforded me the chance of meeting leaders in the field of food microbiology and of observing their analytical and research techniques. The contacts I made were stimulating and broadening. I was always received with cordiality and respect and listened to attentively, and questioned intelligently wherever I went. Part of this high regard probably stems from the fact that the United States is considered to be a leader and protector of democratic institutions. As such, its representatives are looked up to with regard and dignity. A reassuring confidence and understanding is instilled in the minds of those whom we, in turn, look up to for guidance.

As a result of this captivating assignment, I returned to work with enthusiasm and a feeling that the Department of Defense is an enlightened employer, and has great depth of understanding and foresight in its research and development policy.

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FOREWORD

An enigma is encountered with the organism known as Clostridium perfringens. This situation stems from a natural, though innocuous presence of great numbers of this organism--similar to that of Escherichia coli--in the human intestines. Yet, under certain conditions, heat resistant strains of this organism may cause food poisoning. By ingesting food in which this organism has multiplied, varying degrees of distress may affect human subjects, ranging in intensity from moderate enteritis caused by type A to haemorrhagic enteritis necroticans from type F, the latter being very rare. In the least severe cases of the former, patients have recovered in a few hours. By contrast, in the worst cases of the latter, victims have died from dehydration due to severe diarrhea and circulatory failure. Extensive damage to the intestine may be caused by type F, and by type A as well.

Inasmuch as C perfringens type A is a normal inhabitant of the human intestines and is a constant contaminant of soils, water and unprocessed foods proof of its potential action as a food poisoning agent has been hampered. Progress in pinpointing C perfringens as the causative factor is further impeded by the similarity of symptoms attributed to food poisoning from entirely different kinds of organisms.

Results of many outbreaks of food poisoning in the United States are recorded as "etiology unknown." It is not unlikely that these cases may be caused by C perfringens and escape unrecognized as such. Unfortunately, because of the ubiquitous existence of this organism--it is found even in chewing tobacco--a demonstration ofits mere presence in a sample of food is of no significance. Not to overemphasize the ubiquity of this organism it is important to realize that workers such as Elizabeth McKillop found it in every specimen of chicken and sausage that they have examined. And, Louis DeSpain Smith reported its presence in every sample of pepper, whether black, red, cayenne or chili that his laboratory analyzed. Many of these, of course, were non-heat-resistant beta hemolytic strains.

To declare that C perfringens is the cause of enteritis in an outbreak of food poisoning, a diagnostician would have to show that a high proportion of the organisms found were heat resistant, non-hemolytic strains of C perfringens. Furthermore, they should be present in vast numbers--up to one billion or more per gram of specimen to offset the normal incidence of this organism--in all three vehicles: food, vomitus when occurring, and excrement. Consequently most laboratories are not inclined to want to examine foods for the mere detection of C perfringens; and are not prepared for its complete diagnosis. In addition presumptive diagnosis by symptoms, pathology and

cultural characteristics should be corroborated by toxicological investigation. This is a complicated process requiring the services of an experienced toxicologist.

Because of the difficulties in establishing C perfringens as a causative agent in food poisoning no real progress was made in the half century following Klein's observation in 1895 and Andrewes' in 1899 of its presence in outbreaks. Although Larner warned of possible enteritis from this organism in 1922, McClung, in 1945, reported for the first time in the twentieth century in the United States human food poisoning caused by C perfringens. In 1953 interest in this subject was sparked in Great Britain by an exhaustive study made by Betty Hobbs and her associates. Since then reports of food poisoning by C perfringens rose tenfold in Great Britain in six years--from 25 in 1953 to approximately 250 in 1959. This record has not been equalled in the United States because laboratory diagnoses are rarely made. But in all fairness it must be said that Britain's problem in this respect is more acute than ours because of the dearth of household refrigeration there. Cooked meat left unrefrigerated is the principal cause of trouble. Spores of C perfringens that have survived cooking, germinate and multiply in the tepid product. In this connection another anomaly which does not help matters is the fact that food contaminated with C perfringens does not smell or taste bad to any appreciable extent. Meat eaten in this condition is the basis for most of the food poisoning outbreaks in homes and institutions. Nevertheless, similar outbreaks in the United States go unrecognized because C perfringens is not sought routinely in public health laboratories as it is in Britain. Too few fecal examinations for C perfringens are made in the United States to recognize this type of food poisoning.

Obstacles of the aforementioned nature suggest that more application, industry and care be devoted to the analysis of cases of food poisoning heretofore relegated to the category "etiology unknown." With this thought in mind the Department awarded and the recipient accepted this Secretary of the Army Research and Study Fellowship. Complete resolution of the recommendations appearing in this report will require more labor and more time. The fact that the problem has been recognized and basic work undertaken is a step in the right direction.

As a result of this study, some immediately tangible benefits are possible. Since C perfringens is universally distributed, the most practical way of combatting it is through the exercise of increased emphasis on preventive measures. In the main these consist of avoiding the development of a high concentration of this organism through adequate sanitation at every stage of food production and manufacture, refrigeration and cold storage, handling and transportation and cooking and serving; and prompt refrigeration and thorough reheating of leftovers before serving.

Though simple in concept, the importance of these preventive measures cannot be overemphasized, for, considerable margin for improvement in their exercise exists.

Since C perfringens is ubiquitous there is little hope of foods being free from it. They become contaminated through handling, or from utensils, dust, and cutting blocks. Therefore, one must become familiar with the prolific reproductive potential of this organism. Based on a generation time of 20 minutes, C perfringens will increase in numbers 500 fold in suitable food that is held but little more than 3 hours at temperatures from 32 C to 50 C. At this rate the food becomes laden with astronomical numbers of these organisms. Because of the capacity of spores of C perfringens to tolerate the temperature of boiling water up to 5 hours, danger of their germination and multiplication in improperly handled cooked food must be guarded against. Large masses of cooked food in which the spores have survived, if allowed to cool slowly, provide an excellent opportunity for spore germination and multiplication. The same danger prevails with leftover foods being reheated slowly over a low flame. Since the temperature range for multiplication of germinated spores is rather wide, cooked food is not safe unless it is cooled quickly and kept refrigerated. If it is to be maintained hot, the temperature should be over 60 C, for, cells will multiply in apparently hot food below this temperature.

Nothing short of pressure cooking for an adequate time, and at a high enough temperature, will kill spores of C perfringens. However, this type of cooking is not always popular and might be impracticable. Consequently one must cope with this organism as encountered in normal cooking. Since boiling and oven roasting may be inadequate for destruction of the heat resistant spores of C perfringens it is presupposed that normally cooked food will contain them. Obviously, prevention must be directed at keeping these spores from germinating and multiplying. Furthermore, if by chance germination should occur, though initially low in number, and, as such, probably harmless, it is imperative that the cells be prevented from multiplying. Insofar as food poisoning by C perfringens is concerned, the greatest threat lies in multiplication.

A prime prerequisite to prevention of multiplication is the maintenance of food that is to be served, either continuously hot or continuously cold. In this connection hot means over 60 C, cold, under 10 C. Preparation of food several hours, or a day before it is to be served should be discouraged. If this cannot be avoided, the food should be cooked at the boiling temperature. This will assure destruction of the vegetative cells, but not the spores. Then the food should be cooled quickly to refrigeration temperature, and kept refrigerated. This procedure will prevent surviving spores from germinating

and multiplying. In roasting meat, the temperature seldom rises to the boiling point in the center of the cut. Consequently, pieces no larger than 6 pounds should be roasted so that the heat can penetrate quickly, and cooling can be rapid if the meat is to be refrigerated. This is most important for rolled cuts of meat. In reheating cooked food, the boiling temperature should be attained and held for at least 15 minutes to assure destruction of vegetative cells. In the manufacture of baked products containing meats, such as meat pies, Pirie and Harrigan recommend prior heat treatment at sublethal temperatures to induce spore germination, followed by baking to destroy the germinated cells.

If one could be assured that the above precautions would conscientiously be practiced, there would be little need for superior analytical methods.

JOHN E. DESPAUL
Chicago
March 1964

1, 7

RESUME

This report is an account of my activities as a Secretary of the Army Research and Study Fellow. It is presented in five parts. The first part deals with fundamental ~~studies~~ and research accomplished during the academic ~~year 1960-1961~~ at ~~the University~~ of Kentucky. This period included several graduate courses in microbiology and completion ~~of a research project~~. The ~~latter was~~ concerned with the development of a rapid biochemical test for the detection of Clostridium perfringens in foods.

Part 2 was accomplished in a five month period during ~~the~~ latter part of 1961 ~~at the University~~ of Cambridge in England. Here full ~~time was~~ devoted to a ~~research project of an applied nature~~. After investigating the characteristics and heat resistance of a food poisoning strain of C perfringens it was inoculated into many small pieces of raw beef. These were stored at various sub-zero and above-zero temperatures. After suitable time intervals, which extended well beyond ~~the~~ five month period ~~of attendance there~~, the survival of this organism at the various temperatures was determined.

~~Immediately prior to reporting to Cambridge and again right after finishing there~~, a total of four week's time was devoted to laboratory practice with the procedures used in the Food Hygiene Laboratory in London. These procedures, which appear in part 3 of ~~this~~ report, are in constant use in that laboratory for detection of the various food poisoning microorganisms.

~~During the course of this fellowship period, as time permitted and opportunity offered, about~~ a score of research centers was visited, mostly in England, for specific information available at these institutions. A synopsis of each of these visits is presented in part 4 of ~~this~~ report.

Several pertinent scientific meetings were scheduled during the Fellowship period overseas. A listing of the various topics presented at the meetings attended is included in part 5.

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OUTLINE OF ORIGINAL RESEARCH PROGRAM

Nature of proposed research: Detection and identification of pathogenic and spoilage microorganisms in subsistence stores is of importance to assure safe issue of food to troops. Most microbiological methods used in food analysis were originally designed for medical investigations. Clinical methods do not assure complete recovery of all microorganisms present in food. A misleading index of contamination is often the result--some of the microorganisms present are not accounted for. There is a serious lack of technical information pertinent to the identity, concentration, mode of action, and significance of unrecovered food microorganisms. Since pathogens in food are often few in number they are difficult to detect, especially if numerous non-pathogens of similar characteristics are present. Current methods may show no pathogens or food spoilage microorganisms, or only a fraction of them when they exist in numbers sufficient to be a potential economic or health hazard. In order to prove the presence of microorganisms in food it has been customary to promote their growth in selective and differential media. Such media are designed for qualitative detection of specific microorganisms but yield poor quantitative recovery. In some instances laboratory personnel are not sufficiently aware of the existence of this problem. The following abstract from Thatcher substantiates the health hazard in North America stemming from microorganisms not recovered in pertinent foods tested.

"...over a period of 10 years 1500 cases of typhoid were attributed to cheese... staphylococcal enterotoxin in foods remains the most common cause of food poisoning... factors conducive to toxin production are poorly understood... botulism is still more often fatal than not... real health hazards do exist, quite apart from the spoilage problem in foods."

Food analysts should intensify their interest in the complete recovery of significant food microorganisms that are present. Difficulties encountered are due to unknown growth requirements of these elusive microorganisms. It would be particularly useful to relate the microorganisms' specific nutritive requirements to availability of these requirements in the food contaminated by them. Growth may be thwarted but not stopped by the physiologically debilitating forces that microorganisms encounter during food processing and storage. Adaptation to drying, freezing, curing agents, heat, or acidity may produce sufficient changes in food microorganisms to render standard methods incapable of their complete recovery. Basic research should be conducted to understand, isolate, and characterize substances of promise for complete recovery of significant food microorganisms. Abilities of these substances to facilitate recovery of microorganisms from food should

be evaluated. Development of more adequate sample preparation procedures prior to microbiological examination should be undertaken. The formulation of microbiological media specifically suited to food analysis should be attempted. Performance tests should be conducted and adjustments made to assure reliability of results.

Specific objective to be achieved: By studying the nutritive requirements of significant microorganisms currently associated with food poisoning and spoilage, develop media and procedures for the propagation, differentiation, isolation and enumeration, of these organisms. Methods attained will obviate misleading data presently obtained in microbiological evaluation of food, and furnish a basis for establishing standardized procedures.

Potential value to the Department of the Army: Military requirements for wholesomeness of food are generally more rigorous than civilian institutional needs since the damaging factors present are greater. Because of exposure to usage extremes, efforts must be directed to assure that food issued to troops is reliably safe. Present methods for performing microbiological evaluations of food do not always assure that the true condition or safety of subsistence items is being revealed. Microbiological media would be developed to achieve complete recovery of significant food microorganisms. Acquisition of reliable methods would be of practical benefit to military inspection services for detecting sources of danger to wholesomeness of food. Detection of bacterial contamination during initial stages of inspection would be possible. A means for arriving at the true index of the wholesomeness of food intended for consumption by the Armed Forces would be provided. Reliable methods may lead to modification of existing food manufacturing processes and production of subsistence items better able to withstand rigors of military supply lines.

MODIFIED PROGRAM

In implementing this fellowship selected universities were surveyed where studies were being conducted on research problems similar to the one outlined above. The survey revealed that it was advisable to reduce the scope of the broadly stated problem to a narrower, more feasible area. Accordingly it appeared promising to confine the study to anaerobic microorganisms. This choice was based on the small amount of research done on anaerobes. Research studies with anaerobes are fraught with complications experienced in setting up experiments wherein all traces of air must be excluded. Furthermore, qualified faculty in this specialized field of research are rare. Nevertheless it was reasoned that if it were possible to find adequate sponsorship in the information lacking field of food spoilage and food poisoning anaerobes, accomplishment of original research would be more probable than in other more crowded and better informed fields.

Since the final work area was left to the discretion of the recipient and his research advisers, the existing research opportunities to be chosen, consistent with the mutual interest of the Secretary of the Army, were the deciding factors. As a result of the survey the University of Kentucky in Lexington, and Cambridge University in England were found to be distinctly interested in sponsoring the modified problem proposed.

Dr. Ralph H. Weaver, Professor of Microbiology, University of Kentucky, had been developing methods for identification of anaerobic microorganisms over an extended period of time with considerable success. His recent interests were centered on the study of the widely distributed anaerobe, C perfringens. Dr. Weaver offered an excellent program of research with a positive plan of attack well suited as a specific element of the proposed project. His objective was to achieve the rapid isolation and identification of this anaerobe because its significance in food spoilage and food poisoning was not well understood in the United States. The academic year, 26 September 1960 to 26 May 1961 was devoted to this study.

Several leading research institutions in Great Britain have realized the justification for investigating the food poisoning characteristics of C perfringens. It is a continuing problem with them. Publications on this topic are twice as numerous in England as in our country. The research accomplished in Britain is probably higher than indicated by this two to one ratio because the intensity of the investigations in each case in the British institutions is considerably higher than in the United States. So it was fitting that a research sponsor in England was to be sought as a practical complement to the more theoretical study initiated at the University of Kentucky.

Dr. Maurice Ingram, Deputy Director, Low Temperature Research Station, Cambridge University, Cambridge, England, fortunately agreed to participate as sponsor of the second phase of this project. For his part of the investigation Dr. Ingram was to be concerned with the survival of C perfringens and its recovery from meat maintained at several different storage temperatures. This part of the study was done from 17 July to 31 December 1961 at Cambridge under the direct supervision of Dr. Ella M. Barnes.

A third part of the fellowship study, suggested by Dr. Ingram, was to become familiar with the procedures used to detect food poisoning microorganisms in the Food Hygiene Laboratory of the Public Health Laboratory Service, London. Under the direction of Dr. Betty C. Hobbs, detection and identification of the various types of food poisoning microorganisms, and allied research in food hygiene, is the full time activity of Dr. Hobbs' laboratory. The fellowship objective here was to acquire the knowledge and experience necessary to initiate a food hygiene program in the Defense Subsistence Testing Laboratory similar to that in London's Central Public Health Laboratory. Two fortnightly periods, one before going to Cambridge and one after finishing there, were devoted to actual performance of the tests in question. Specific information concerning procedures, stock cultures, media, materials, and reagents needed to test for food poisoning microorganisms in the DSSC Laboratory in Chicago was to be obtained there. It was anticipated that the establishment of a food hygiene program in the DSSC Laboratory to test for the possible presence of food poisoning microorganisms would have a wholesome effect on suppliers and would provide greater assurance for safe rations for the Armed Forces.

During the fellowship period in England several laboratories and research institutions where interests coincide with those in the DSSC Laboratory were profitably visited. Also during this period pertinent scientific meetings were attended where theoretical and practical developments were discussed, demonstrations observed, and important personal contacts made.

INTRODUCTION

Versatility of C perfringens: Many hundreds of technical papers have appeared in divers scientific publications throughout the world on the characteristics and activities of C perfringens. The reasons for this great interest stem from the fact that this organism is probably the most widely spread pathogen in existence; and it is indeed a most versatile microbe considering its many unusual activities. It is present in soils of all lands; in all bodies of water; in dust, refuse, and sewage in all surroundings; and in the intestines of animals and man everywhere. A factor contributing to its ubiquity is the resistance of its spores to drying and some strains to heat. C perfringens is perhaps best known for its insidious attack of the deeper wounds in animals and man, causing gangrene. No matter in what part of the world the battlefield or scene of accident involving bodily injury may be, C perfringens is lurking about ready to spring into its destructive action. Besides the gangrene of deep wounds, other diseases caused by C perfringens in humans include fatal infections following abortions, pathogenesis in obstructed intestines or blocked arteries of the kidneys, serious post operative infections, and food poisoning. Veterinary diseases, often fatal, caused by this organism include dysentery of newborn lambs; enterotoxemia of sheep and goats; acute hemorrhagic enteritis of baby calves; hypoglycemia and necrotic enteritis in piglets; and acute grass sickness in horses. At least twelve toxins, identified with twelve letters of the Greek alphabet, are variously produced by the six types of C perfringens. These six types are designated by the letters A, B, C, D, E, and F. Each type produces one to six or more of these toxins. These organisms wreak havoc in any tissues they invade, inasmuch as nine of the twelve toxins are lethal.

An entirely different function of C perfringens has been its use as a leavening agent in certain provincial bakeries for the production of a special kind of bread. The gas forming characteristic of this organism is thus used to leaven a type of bread called "salt rising bread" in much the same way as yeast is used in conventional bakeries. Apparently no ill effects have been reported from the use of such bread starters containing C perfringens in the inoculum, although the organism is not necessarily destroyed in the baking. Betty Hobbs found such a starter to be composed of mixed organisms with small numbers only of C perfringens.

C perfringens as a food poisoning agent: Since about 1900, microbiologists have been presenting evidence that C perfringens is the causative agent in numerous cases of mild to severe enteritis. Such diagnosis is ideally based on laboratory demonstration that the organism is present in three distinct environments: a) in the contaminated food that was eaten, b) in the

vomitus* of the patient, and c) in the excrement. However, acute gastroenteritis accompanied by abdominal pain and diarrhea can be caused by C perfringens without the manifestation of nausea and vomiting. In cases of food poisoning caused by organisms other than C perfringens evidence obtained for the organism identified may be substantiated by production of typical symptoms with animal feeding experiments. However, it has not been possible to induce such symptoms experimentally in any laboratory animals that were given food contaminated with C perfringens.

Some of the information concerning C perfringens is controversial. This has created doubt as to its ability to cause food poisoning. Since C perfringens is found in the intestinal tract of nearly all healthy persons from infancy to old age, some observers believe that it is not the true causative agent in digestive disturbances of food origin. They point to the use of this organism as an indicator for measuring the fecal pollution in water analysis in Europe, similar to the use of E coli in the United States. Since C perfringens is so extensively distributed in nature, as pointed out by numerous authors, but most recently by Dr. Strong of the University of Wisconsin, it is apt to be a probable contaminant of most foods.** Indeed it has been found in practically all fresh food products and in many manufactured food items where the heat resistant spores of the food poisoning strains may not be affected by the processing. Therefore, since C perfringens is a natural contaminant of food and is present in the intestinal tract, its identification in food and feces samples in outbreaks of food poisoning has been challenged as conclusive evidence that it is the causative agent of the outbreak.

If consistent symptoms with human volunteers would follow upon ingestion of food known to be contaminated with this organism, significant conclusions could be drawn about its food poisoning characteristics. The possibility of grave consequences has limited such feeding experiments with human beings to a few rare occasions since this organism was discovered in 1892.

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*No up-to-date information is available on the vomitus of patients affected with food poisoning due to C perfringens. The incubation period is long and there is no proof that a toxin or toxoid system is involved.

** It must be borne in mind that certain strains of C perfringens, those most implicated in food poisoning, have heat resistant spores. Consequently, a high proportion of the contaminants will be non-heat resistant beta hemolytic strains.

These few studies have given conflicting results. In the first recorded instance, at the turn of the century, one volunteer had eaten cultures of the organism without ill effects. Another, reported by McClung in 1945, upon eating food contaminated with C perfringens developed symptoms similar to those of the victims of a food poisoning outbreak. In 1952 Osterling produced food poisoning symptoms in several persons who had eaten food that was experimentally contaminated with C perfringens. The same food, uncontaminated, or the bacteria free filtrate of the contaminated sample, or the recooked contaminated sample caused no discernible illness. Since the sterile filtrates were not toxic, Osterling concluded that the symptoms were caused by infection with the live organisms and not the filtrable toxin. None of his tests with apes, cats, dogs, or pigs gave positive results, indicating that the food poisoning characteristic of C perfringens is limited to human beings. Betty Hobbs in 1953 conducted feeding tests with a few volunteers. Some of these experienced mild symptoms, while others did not. As with other observers her results with animals were negative. On the other hand in a more extensive study, Dack in 1954 conducted feeding experiments with four strains of C perfringens on 32 volunteers, but obtained no symptoms of food poisoning. Observers later agreed that ingesting the cultures alone did not produce illness; but eating food that had been inoculated with the organism and incubated for a suitable period of time caused typical symptoms. In such an experiment with 41 volunteers Dische and Elek in 1957 produced symptoms that were identical with those in naturally occurring outbreaks. They stressed that a proper, heat-resistant, food poisoning strain of C perfringens is required. If indefinite or negative results are obtained they suggested that the virulence of the strain may be lost. This could explain the findings of the observers who obtained negative results.

Some of the information presented above may weigh heavily against serious consideration of C perfringens as a food poisoning agent. However, in repeated outbreaks of food poisoning the failure to find other species in significant numbers, and the persistence of high counts of C perfringens in food and excrement has nevertheless intensified interest in the subject. The solution to the problem would seem to involve resolution of differences in findings by various investigators and integration of essential factors. Isolation of C perfringens of one serological type from a high proportion of fecal specimens from patients would be most significant. This requirement is important, and should be stressed, particularly in the United States, for, correct diagnostic information without investigation of fecal specimens is not possible. In recent years the possibility of the symptoms under consideration being caused by bacteria other than C perfringens has largely been ruled out. Furthermore, concrete evidence is being presented establishing the finding that this organism is the actual agent causing the food poisoning when so claimed. The isolation of C perfringens from food and excrement, not

only in far greater numbers than can account for its incidental presence, but also the high proportion of stools found to be positive for one serological type in any specific outbreak has aided materially toward reducing the number of cases otherwise categorized as "etiology unknown."

REPORTS OF THE SEVERAL PHASES OF WORK ACCOMPLISHED

FROM 26 SEPTEMBER 1960 TO 19 JANUARY 1962

in fulfillment of a

Secretary of the Army's

Research and Study Fellowship

awarded to

John E. Despaul, Assistant Chief
Laboratory Division
Directorate of Technical Operations
Defense Subsistence Supply Center
1819 West Pershing Road
Chicago 9, Illinois

P A R T I

Report of project

DEVELOPMENT OF A BIOCHEMICAL TEST FOR *Clostridium perfringens*

Accomplished during the period

26 September 1960 to 29 May 1961 at the

Department of Microbiology,

University of Kentucky

Lexington, Kentucky

in connection with a

Secretary of the Army's

Research and Study Fellowship

by

John E. Despaul, Assistant Chief
Laboratory Division
Directorate of Technical Operations
Defense Subsistence Supply Center
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RESEARCH AND STUDY AT THE UNIVERSITY OF KENTUCKY

Investigations concerned with the detection and identification of C perfringens in food poisoning outbreaks are often hampered by the lack of rapid and reliable methods for this purpose. Available techniques are complicated to the extent that this organism is not routinely sought in many laboratories that should be concerned with its detection. Gibbs and Freame have commented, "There is no satisfactory selective medium for detecting and counting all the Clostridia in foods. . . . " Because of these obstacles practically every analytical procedure that appears in current compendia, or is in vogue in most laboratories, has the author's or director's peculiar interpretation of how the presence of this organism is to be substantiated.

This investigation was undertaken to fill the need for a simple, reliable direct test to determine the presence of C perfringens for screening purposes as a preventive measure in food laboratories, and for quick diagnostic requirements in food poisoning outbreaks.

Summary: During the academic year 26 September 1960 to 29 May 1961 the recipient was enrolled at the University of Kentucky in three courses each semester. These included advanced general bacteriology; virology; immunology; history of bacteriology; biochemistry; and seminar. The first three of these courses included laboratory experiments. The remainder of the time was utilized on research concerned with the development of a rapid biochemical test for the identification of C perfringens. This test is based on the differential action of Clostridia on amino acids and amides. The enzymatic ability of Clostridia to deaminate specific amino acids and amides was studied by testing these organisms for the production of ammonia in a buffered substrate that is nitrogen free except for the amino compound being tested.

Cells were grown in 90 ml centrifuge tubes in a clear broth medium consisting of yeast extract, trypticase, and phytone under hydrogen in a Brewer jar at 37 C. To wash the centrifuged cells free of nitrogenous compounds they were resuspended in a buffered glucose solution and recentrifuged. This buffer served as a protective environment during centrifugation.

Substrates consisting of 1 per cent solutions of specific amino acids or amides in buffered glucose were set up in test tubes size 13 x 100 mm. A suspension of the washed cells was added to these tubes to a turbidity approximating a Number 4 MacFarland nephelometer tube. These substrate tubes were incubated under hydrogen in a Brewer jar at 37 C and tested for the production of ammonia. Ammonia was detected either by Nessler solution, or

with neutral red indicator, or by Hansen's method. As the testing progressed Hansen's method was used exclusively since it was found to be more specific for ammonia than the other two methods. In all cases two controls were run using buffer and organisms only, and buffer and amino compounds only.

About 30 amino compounds were tried with C perfringens. Positive reactions were obtained with five of them namely, 1-arginine monohydrochloride, 1-asparagine, glutamine, dl-serine, and dl-threonine. The reactions of these five compounds were studied with about 20 species of Clostridia in order to develop a scheme of analysis for rapid detection of C perfringens as a species.

Handicaps inherent in microbiological methods: Certain difficulties, not encountered in physical or chemical tests, are inherent in microbiological procedures. Since morphological characteristics of entities so small as bacteria are inadequate for their identification, other features must be used for their determination. Time consuming biochemical and cultural properties must be developed and analyzed for the identity of microorganisms. Growth, and production of biochemicals, together with time required for isolation and analysis often take too long to be of value in bacteriological investigations.

Available procedures are complex, requiring highly skilled personnel to perform them. Not one, but usually a battery of tests must be used to identify an organism, since several characteristics of some bacteria may be identical with others. Sufficient characteristics must be determined to the extent that some will be found that will differentiate one organism from another. This necessitates elaborate, large scale laboratory operations to keep the relative cost per test down to a reasonable figure. In small laboratories where manpower is limited the odds are against undertaking procedures that are requested infrequently. The expense of preparing little used media and reagents each time they are needed would be prohibitive. Efforts therefore are made to develop test procedures that will conserve labor, equipment, and materials and will provide results simply, quickly, and dependably. Only if they measure up to these requirements for economy and efficiency will new tests be universally adopted in small laboratories and where so-called routine test work is done. Development of such a test for C perfringens was the aim for the portion of this fellowship undertaken at the University of Kentucky. Attempts were made to establish ideal incubation conditions. Bearing in mind the fact that the smaller the inoculum used the longer would be the lag phase, immediate steps were taken to overcome this tendency. Small amounts of a select medium were used into which a large inoculum was introduced into small tubes to permit the organisms to start growing rapidly in the logarithmic phase with a minimum lag phase. The

objective was to produce as quickly as possible sufficient concentrations of biochemical products to be detectable with sensitive reagents.

As an initial reference guide the properties of C perfringens were tabulated as shown below.

Characteristics of C perfringens:

Habitat

Soil, feces, sewage, gas gangrene, air, dust, bodies of water, and foodstuffs, particularly meat.

Morphology

Shape: Short thick rods
Size: 2 to 4 u long and 0.8 to 1.5 u wide
Spores: Oval, central to eccentric not swelling the cells

Flagella: None	Capsule: Present
Motility: None	Gram staining reaction: Positive

Cultural Characteristics

Surface agar colonies:	Semiconvex, semitranslucent, glossy, entire margins, overnight incubation 1 to 3 mm diameter
Deep agar colonies:	Lenticular or shaped like apostrophes
Colony variation:	Smooth, mucoid, or rough depending on age of culture and type of medium

Fermentation Reactions

Glucose:	Acid and gas	Raffinose:	Acid and gas
Fructose:	Acid and gas	Starch:	Acid and gas
Galactose:	Acid and gas	Dextrin:	Acid and gas
Mannose:	Acid and gas	Inulin:	Variable
Xylose:	Acid and gas	Glycogen:	Acid and gas
Lactose:	Acid and gas	Glycerol:	Variable
Sucrose:	Acid and gas	Mannitol:	Not fermented
Maltose:	Acid and gas	Inositol:	Acid and gas
Trehalose:	Acid and gas	Salicin:	Variable

Growth Factors

Oxygen: Tolerates about 3 per cent of oxygen content of air
Temperature: Optimum 37 C, no growth beyond 50 C
Nutrients: Requires 13 amino acids, vitamins, adenine, uracil, minerals
Atmospheric nitrogen: No fixation

Biochemical Tests

Hydrogen sulfide: Forms black colonies in suitable medium
Indole: Not produced
Nitrate: Reduced to nitrite
Sulfite: Reduced to sulfide

Protein Media Reactions

Litmus milk: Acid, profuse gas, casein coagulated, not digested
Gelatin: Liquefied and blackened
Coagulated albumin: Not liquefied
Coagulated blood serum: Not liquefied
Brain medium: Not blackened or digested
Egg-meat medium: Considerable gas, no digestion

Hemolysis

On horse blood agar many strains produce zones of beta hemolysis. Some strains produce partial hemolysis. Food poisoning strains produce no hemolysis. Colonies of non-food poisoning strains of type A are surrounded by narrow zones of complete hemolysis. Food poisoning strains of type A may be non-hemolytic or faintly alpha hemolytic after 24 hours incubation on horse blood agar. Although hemolysis of food poisoning strains may increase slightly on standing, it cannot be described as complete. Types B and C on sheep blood agar may have large zones of hemolysis. Type F strains do not have an inner zone of complete hemolysis.

Exotoxins

The six types of C perfringens, A, B, C, D, E, F produce variously one to five or more of 12 toxins which are designated by Greek letters. Type A causes human gas gangrene. Non-hemolytic strains of type A cause food poisoning. Type B causes lamb dysentery and enteritis of goats and sheep. C causes struck in sheep and enteritis in calves, lambs, and piglets. D affects the adults of these animals. E causes enterotoxemia in animals. Type F causes enteritis necroticans in man--a severe type of food poisoning which is very rare.

Special Nagler Test

C perfringens on lactose egg yolk milk agar of Willis-Hobbs containing neutral red produces opalescence which is inhibited by alpha antitoxin. Pearly layer is not produced. Lactose is fermented and no proteolysis occurs--all on the same plate--diagnostic of C perfringens.

Characteristics previously investigated: Some of these characteristics had been investigated by previous workers at the University of Kentucky. Rapid methods for the detection of carbohydrate fermentation, and for the simultaneous detection of gelatin hydrolysis and indole formation to differentiate several species of Clostridia were described by Kaufman and Weaver in 1960. These authors also published on the use of neutral red fluorescence for the identification of colonies of Clostridia. Further work on fluorescence as a means of selection of Clostridia was reported by Fitch in an unpublished continuation of Kaufman and Weaver's study. Similarly Gordon wrote a paper on his observations of the utilization of natural proteins in media for identification of Clostridia.

Action of Clostridia on natural proteins: Dr. Weaver's initial plan for the Fellowship research called for resumption of Fitch's investigation of fluorescence and Gordon's work on proteins. These students developed the tests for identifying the Clostridia as a group. Further development of the concepts they investigated was expected to distinguish the individual members of the genus. In view of the proteolytic nature of the Clostridia in general, an investigation of their relative biochemical action on proteins appeared both challenging and feasible. Therefore, following up Gordon's research with natural proteins was considered first. The object of this investigation was the development of the use of undenatured proteins in culture media for rapid identification of the individual members of the genus Clostridia. Enrichment of the medium with the correct natural protein would foster quick growth. Various proteolytic enzymes secreted from the organisms at different rates would yield characteristic products of hydrolysis from the undenatured proteins. Reacting with sensitive reagents, these products would enable identification of the Clostridia in question, in proper succession. Thus, at an expected period in the scheme of analysis, C perfringens would make itself evident.

Egg albumin, casein as sodium caseinate, and beef serum were available in dry, soluble form. Raw egg white was also used. Gordon experienced considerable difficulty in sterilizing the albumin solutions either by heat or by the use of Seitz type filters. He also noted either very little or inconclusive hydrolysis of the albumin medium inoculated with heavy suspensions of the organisms under consideration, which he incubated anaerobically in a hydrogen

atmosphere or under a cap of agar. His experiments with suspended coagulated albumin, and formalin treated albumin, presented further difficulty in uneven dispersion and precipitation with the aid of charcoal. Switching to raw, chicken egg albumin Gordon spotted an agar medium containing 0. 5 per cent of this natural protein with different species of Clostridia. He noted no change in the plates that had been previously heated at 90°C for 10 minutes to denature the protein. Unheated plates showed more response when incubated 72 hours than after 24 hours. Since it was not practical to sterilize the raw egg white, the results of hydrolysis were considered inconclusive in view of the longer incubation period even though a very heavy inoculum was used.

Various forms of experimental substrates using soluble casein or beef serum were designed by Gordon in further natural protein studies. Heavy inocula, with several species of Clostridia, were used in tubes, or spotted on plates. Ninhydrin was the most sensitive of several reagents used to detect hydrolysis. In many cases the degree of hydrolysis was similar for the separate organisms studies. It was therefore difficult to interpret the results obtained and, consequently, not possible to distinguish, by these color tests, one organism from another. Unfortunately, false positive results were obtained for the perfringens strains. This was attributed to the lack of specificity of the Ninhydrin reagent since some color was obtained from the uninoculated natural protein control tube itself. After careful review of all the details and possibilities presented in this experimental work it was decided that undenatured proteins could not be used to advantage in preparation of culture media for rapid identification of the Clostridia.

Fluorescence of Clostridial colonies: The possibility of utilizing the principle of neutral red fluorescence reported by Kaufman and Weaver was next explored. In the study for his dissertation Kaufman found that members of the genus Clostridia, grown in a rich medium containing neutral red, produce a fluorescence that is visible in ultraviolet illumination. When growth is abundant this fluorescence can be seen in ordinary light. Such fluorescence was more obvious and intense in ultraviolet than in daylight. If fluorescence of the desired colonies could be relied upon, it would be possible to separate the Clostridia from facultative anaerobes as well as from obligate anaerobes of other genera. A clue was therefore at hand to adapt this characteristic to quick identification of colonies of Clostridia and adjust conditions to make it selective for C perfringens. This could conceivably be done by determining the shortest possible time required to detect fluorescence for each of the Clostridia species and standardizing the conditions needed to duplicate the experiment. Fluorescence, appearing at a predictable time interval, could be correlated with the proper species expected. If successful, the technique could be further improved by reducing it to a microanalytical test. Thus, by means of slide cultures, colonies could be detected more quickly with the microscope

using a method of growing anaerobes similar to the one devised by Steinkraus in 1949 and Harbour in 1954.

From the middle to the end of the first semester experiments were conducted to further the fluorescence work initiated by Kaufman and Weaver and continued by Fitch. At the suggestion of Dr Weaver the study was performed in conjunction with Dr Dudley C Martin of the Agricultural Experiment Station, University of Kentucky. Dr Martin was engaged in food research of interest to the Department of the Army since it was concerned with a contract awarded to his station by the Armed Forces Food and Container Institute, Number 87 R & D Natick, of which Dr Fred Heiligman of the Food Radiation Preservation Division was project officer. Among other contaminants Dr Martin desired to determine whether or not any Clostridia species were present in certain food samples held in storage and preserved only by irradiation. He was willing to apply and improve, if possible, the fluorescence techniques of Kaufman and Weaver, and Fitch, to the analysis of his food samples for Clostridia. Although Dr Martin's objective was a complete microbiological analysis of the samples in question our observations will be limited to the experiments we conducted to detect Clostridia through the use of fluorescence.

Four series of tubes and plates from each of 30 samples of irradiated sweet corn were set up. There were 30 tubes and 30 plates for anaerobic analysis and the same number for aerobic tests. Kaufman and Weaver's procedure was used in preparing the 30 plates for observation of fluorescence. This consists of a Petri dish with three layers of liver veal agar containing a concentration of 0.004 per cent neutral red. The bottom layer of 12 ml or less is allowed to solidify. On top of this is poured an inoculated layer of the same medium. After solidification, this in turn is capped with an amount of the medium equal to the bottom layer. The plates are incubated at 37 C in a Brewer jar in an atmosphere of hydrogen. After incubation periods of 18 to 48 hours the uncovered plates are examined in total darkness on a black background under ultraviolet light from a "Mineralight" lamp. Colonies of Clostridia should be recognized after 18 to 24 hours of incubation. As seen with ultraviolet illumination they should be discernible by a zone of golden yellow fluorescence surrounding each colony. After 24 to 48 hours a yellow fluorescence should be noted with ordinary illumination that is quite distinct from the pale yellow color of an alkaline reaction.

Counts after 48 hours incubation revealed 12 plates with no colonies, 12 with more than 100 submerged colonies, and the remaining 6 plates with 1, 2, 2, 5, 7, and 43 colonies respectively in the middle layer. All colonies appeared to be negative to fluorescence under ultraviolet light. However the medium itself appeared to fluoresce rather intensely under the influence of the ultraviolet. Kaufman and Weaver's procedure specified the use of neutral

red of the National Aniline Division, Allied Chemical Corporation. Instead, an apparently equivalent neutral red manufactured by Matheson, Coleman and Bell was used.

In the next experiment to check the fluorescence technique two sets of ten plates each were prepared according to the Kaufman-Weaver procedure. One set contained neutral red made by National Aniline, the other by Matheson-Coleman-Bell. Inoculations of each set were made in duplicate using five different organisms. Two of these included the standard stock cultures of C perfringens. The other three were taken from select colonies of representative corn samples that had shown positive contamination with anerobes. After 20 hours of incubation the plates were examined. Two types of ultraviolet light were used. One was a General Electric germicidal lamp having a short wavelength of 2600 Angstroms. It produced a reasonably good fluorescence with the University of Kentucky C perfringens Nr 1 strain. This fluorescence was partly masked by fluorescence of the agar medium. Our control strain Nr 2 did not grow. Also, no growth occurred with one of the three organisms picked from the corn samples. Growth occurred with the inoculum from the other two samples but the colonies did not fluoresce. The fluorescence obtained with the U of Ky C perfringens Nr 1 strain was much poorer when the illumination came from a "Mineralight" bulb having a wavelength of 3600 Angstroms. This lamp was also used with a Black Raymaster Bulb of 3600 Angstrom wavelength made by George W Gates Co. These longer wavelength lamps were supposed to give better fluorescence than short wave bulbs. All the plates that had no colonies produced strong fluorescence from the surface of the agar. No difference could be noted in the fluorescence observed from the agar or the colonies regardless of whether the dye used was the National Aniline brand or Matheson-Coleman-Bell. It was interesting to note that when a section of the agar was removed from the Petri plate it fluoresced under the ultraviolet light throughout its three dimensions, making it look like a glowing piece of cheese. Of interest also was the fluorescence of the C perfringens colonies in bright daylight. The glow appeared to be as brilliant in daylight as under the ultraviolet light.

Because of interference from fluorescence of the agar medium it was decided in future experiments to minimize this undesired fluorescence by reducing the thickness of each of the three layers. Instead of 12 ml of medium for the top and bottom layers and 10 for the middle the volume in each layer was reduced to half this amount. Accordingly a set of 16 plates was prepared. Ten of these were inoculated with anaerobic colonies isolated from 10 different corn samples, 4 were from stock cultures of C perfringens, and 2 were uninoculated blank controls. In observing the plates after 18 hours the results were rather unimpressive with whatever ultraviolet light source used. Although the thickness of the three layered plates was at a satisfactory minimum

the fluorescence from the medium itself was quite appreciable. Under ultraviolet light colonies in all four C perfringens plates were visible with what might be called fluorescence, but it was not distinct nor convincing. With ultraviolet illumination from the germicidal blub the medium did not fluoresce as greatly as when the "Mineralight" was used. The colonies appeared as milky masses. This may have been the image created by the visible light also generated by the bulb. In other words the colonies were visible by ordinary illumination, and the fluorescence came from the medium. Halos around the colonies where the neutral red color had been changed by metabolites from the organisms were also visible. None of the colonies that developed from the corn sample inoculations fluoresced. However, a fluorescent glow occurred in the medium of these plates as well as in the blank control plates that were not inoculated.

Investigation of interfering fluorescence: An effort was made to eliminate the interfering fluorescence of the medium by varying the concentration of the neutral red dye in order to arrive at the optimum level. Neutral red concentrations ranging from 0.0005 to 0.0100 per cent were used in preparing ten three-layered plates for the fluorescence test. The center layer of each plate was inoculated with the same strain of a stock culture of C perfringens. After 20 hours of incubation the plates were examined for fluorescence. A strong background fluorescence appeared in the medium of all the plates regardless of concentration of the neutral red. Some fluorescence of individual colonies was also discernible. This was more evident with the Black Raymaster lamp than with the short wave germicidal bulb. It was concluded that the dye concentration is not of great significance.

Another experiment was done varying only the pH of the medium. The undesirable strong background fluorescence persisted in all ten plates that were prepared for this study. Therefore, the fluorescence of the medium was not a function of pH. Fluorescence of colonies on a control plate inoculated with Clostridium perfringens was masked by the fluorescence of the medium as observed in previous tests.

Since the problem of background fluorescence remained unsolved, it was speculated that the agar itself might be the cause of the interference. To investigate this point every kind of agar medium that had been in use in the Department of Microbiology was collected and tried by the Kaufman-Weaver test. Various degrees of fluorescence, with all of the agar-containing media investigated, was obtained, proving that the fluorescence of agar in this test is an interfering factor.

Effective and selective observations of young colonies of Clostridia could be expected through the facility of fluorescence if several basic problems were

solved. In order to duplicate and enhance Kaufman and Weaver's procedure for detecting Clostridia, a study of the conditions affecting the quality of fluorescence should be made. Factors such as the influence of reflected and transmitted light on the fluorescence produced require consideration. Difficulties involved with glare, thickness of medium, and local dehydration at the surface of the agar would have to be resolved. A standard light source to provide intense, precise fluorescence and sharp outlines of colonies is needed. From the observations made, and in view of the problems presented, no further experiments were performed with fluorescence. A fundamental investigation of the fluorescent properties of the Clostridia in neutral red at various wavelengths was therefore considered to be beyond the scope of this fellowship study. At this point Dr Martin terminated his association with the project since his interest was to last only until the analytical work in connection with his irradiated samples was completed.

Enzymes of C perfringens: During the second semester at the University of Kentucky the approach to this study was changed. The time available for research was devoted to seeking a highly reactive enzyme of C perfringens as a basis for the rapid detection of this organism. A search of the biochemical literature concerned with enzymatic reactions of the Clostridia revealed some attractive information from which ideas could be gleaned. Hughes and Williamson discussed the glutaminase of this organism; Bidwell its proteolytic enzymes; Macfarlane its lecithinase; Brooks its urease and Oakley, Warrack, and Van Heyningen its collagenase. Dolin reviewed the diphosphopyridine nucleotide oxidase and cytochrome reductase of C perfringens and also its peroxidase and diaphorase. Rogers pointed out enzyme systems of this anaerobe which cleave large molecules such as gelatin, deoxyribonucleic acid, and chitin. Numerous other enzymes of C perfringens ferment specific carbohydrates, and several decompose peptides and amino acids. From this array of biochemical catalysts it appeared advantageous to concentrate on the latter group of enzymes that attack amino acids and amides. Fewer complications in detecting the byproducts were anticipated with this group than with other enzyme systems.

Burrows, Gordon, and Porter stated that amino acids are vigorously attacked by the obligate anaerobes. Often the hydrolytic products include organic acids, such as butyric and acetic, along with carbon dioxide, hydrogen, and ammonia. Arginine is decomposed by Clostridium perfringens, the fermentation products being ornithine, carbon dioxide, and ammonia. In fact, valuable enzymatic methods, with preparations from Clostridium perfringens acting on the substrate, have been introduced by Krebs, and also by Archibald, for the quantitative determination of asparagine, glutamine, and glutamic acid. Furthermore, Gale reported the quantitative estimation of histidine; while Meister, Sober and Tice described the degradation of

aspartic and glutamic acids and their determination by enzymatic decarboxylation with this organism. Decomposition of amino acids by the Enterobacteriaceae was used for classification purposes by Lysenko and Sneath. These were some of the more important of many amino acid degradation studies published.

The decision to search for an enzyme that would react with an amino acid or amide was a fortunate choice since, in recent years, these compounds have been standardized and would be available in highly purified form at relatively reasonable prices. The choice was also prompted by the fact that ammonia was one of the products common to all of the reactions that would be positive, and, it was believed, could be detected readily. It was planned to start by adding one of about 30 of these nitrogen compounds, in turn, to a standard anaerobic medium minus the usual nitrogen source. This would tell whether, and how quickly, the corresponding enzyme of C perfringens would deaminate the supplement. For example the arginase of the organism would decompose supplemented arginine and the ammonia produced would be found in the medium as an ammonium salt. Since the ammonia produced was to serve as an index of the reaction, a sensitive test for its detection was to be decided upon. In coping with other anaerobes having enzymes identical with C perfringens the same study would have to be accomplished systematically with each of 30 or more nitrogen compounds at molecular level to determine the cross reactions that would occur with these organisms. Approximately 20 species of Clostridia in the Microbiology Department were considered to be about as good a collection of these organisms as was available anywhere; and as such would constitute a rather thorough study. Our experiments were to reveal how the reactions of the other species of Clostridia would compare with that of C perfringens after the most favorable conditions for the latter had been developed.

Liberation of ammonia from various media by C perfringens: In the first experiment undertaken in this project twelve standard anaerobic broth media were inoculated with a strain of C perfringens and incubated in a hydrogen atmosphere at 37 C. The objective was to find out if one of these media would be suitable for supplementation with an amino acid or amide as planned. If after incubation the organism grew well and produced no ammonia in the substrate, the medium could be used as a base for adding the nitrogen compounds to be tested. The different broths tried in this manner were made from:

a) yeast extract
b) trypticase
c) phytone
d) veal infusion
e) heart infusion
f) brain heart infusion
g) cooked meat infusion
h) egg meat medium
i) peptone colloid medium
j) liver veal infusion

k) brain liver heart infusion
l) yeast trypticase phytone medium

All of these media gave positive reactions for ammonia after 48 hours incubation at 37 C when tested with Nessler solution. Therefore the conclusion was readily arrived at that it would be necessary to develop a basic medium for the proposed study. Obviously the medium would have to be made without any nitrogen compound in it so as to preclude the possibility of ammonia being produced from any other compound than the nitrogen supplement being tested. This observation led to a further search of the literature to investigate the composition of elementary media used in similar metabolic studies.

Degradation of arginine by Streptococci: One of the early investigations of the degradation of an amino acid by bacteria in a culture medium was reported by Niven, Smiley and Sherman in 1942. Arginine, added to a basal medium, was hydrolyzed by certain Streptococci with the production of ammonia. This finding was considered to be of value as an aid in differentiating the species of Streptococci that have the ability to produce ammonia in such a medium from those that do not. These authors used a medium consisting of 0.5 per cent yeast extract, 0.5 per cent tryptone, 0.2 per cent phosphate buffer and 0.05 per cent glucose. This was supplemented with 0.3 per cent d-arginine monohydrochloride. As a control the same medium minus the arginine was used. After inoculation and incubation, ammonia was detected with Nessler reaction or by micro-Kjeldahl distillation. A low concentration of glucose in the medium yielded more ammonia than greater amounts because of probable inhibition from increased acid production when more glucose was used. The optimum pH was 8.5. In the same paper the authors reported a good correlation between the production of ammonia from the same medium containing either peptone or tryptone with or without arginine being supplemented. In other words the control also was positive for ammonia. From this finding it could perhaps be concluded that arginine might be the substance responsible for the production of ammonia from the peptone or tryptone. However, the suggestion that such a medium with arginine added could be used as a differential test for microorganisms was thought to be of doubtful value.

Degradation of amino acids by C perfringens: In a fundamental analysis of the metabolism of amino acids by C perfringens Woods and Trim found that this organism attacked five of 21 amino acids available at that time. C perfringens, grown under the best conditions then known, produced ammonia from serine, cystine, cysteine, threonine, and arginine. Here, too, the authors avoided a high concentration of carbohydrate in view of the known inhibitory effect of sugars on the production of amino acid hydrolyzing enzymes by these organisms. Instead of a medium containing complex nitrogen sources,

Woods and Trim used elementary substrates of each amino acid separately in a phosphate buffer at pH 7.2. Washed suspensions of the organism were used as the inoculum and controls were set up in each case without the amino acid substrate. Their paper included detailed studies of the kinetics of the hydrolysis of these amino acids. Additional research by Chargaff and Sprinson described improved conditions for deamination of serine and threonine by several organisms including C perfringens.

Essential amino acids and other nutritional requirements of C perfringens: Considerably more information on the experimental conditions required for efficient study of the action of C perfringens on amino acids was available from the work of Boyd, Logan, and Tytell done at the University of Cincinnati. This group of workers determined the detailed growth requirements of C perfringens to the extent necessary for the use of this nutritionally sensitive organism for the quantitative assay of many amino acids. To produce large inocula of the organism for numerous tests of its capacity to react with amino acids vigorous young cells would be required, and could be grown consistently only if the ideal nutritional requirements were known. Such were the conditions established by the Cincinnati research team who, incidentally, were working in collaboration with the Department of the Army. Dense growth was obtained at pH 7.2 in 16 hours at 45 C in a medium of known chemical composition which contained ascorbic acid as a reducing agent. It consisted of 19 amino acids; the nucleic acids uracil and adenine; glucose; six salts; and four vitamins. The latter were biotin, calcium d-pantothenate, riboflavin, and pyridoxamine. Although their experiments showed that 13 amino acids were essential it was expedient to use a hydrolysate made by digestion of casein with trypsin. This digest contained 19 amino acids which included the 13 essential ones. For convenient reference the amino acids and salts composing the basal medium are listed below. Of those listed, alanine, aspartic acid, glycine, hydroxyproline, lysine, and proline are not essential.

DL - Alanine	L - Leusine	DL - Valine
D - Arginine	L - Lysine	$MgSO_4 \cdot 7H_2O$
DL - Aspartic acid	DL - Methionine	$FeSO_4 \cdot 7H_2O$
L - Cystine	L - Proline	$MnSO_4 \cdot 4H_2O$
Glycine	DL - Phenylalanine	NaCl
L - Glutamic acid	DL - Serine	K_2HPO_4
L - Histidine	DL - Threonine	KH_2PO_4
Hydroxy-L-proline	L - Tryptophan	
DL - Isoleucine	L - Tyrosine	

The pH was adjusted to 7.2.

Under these growth conditions, no known toxins of C perfringens were formed in this medium. The omission of any one of the 13 essential amino acids, or

vitamins, or nucleic acids, in the otherwise complete medium, resulted in practically no growth of the organism. Omission of any of the amino acids that are not essential was of no significance. Of the salts listed, magnesium and iron are essential requirements as pointed out by Webb. The buffer salts keep the pH high during growth. Below pH 5.0 growth is meager and ceases at pH 4.5. In autoclaving the medium glucose must be sterilized separately by heating at 100 C for 20 minutes. It is then added to the other sterile substances. Otherwise carmelization with production of growth inhibitors occurs. Ascorbic acid or some other suitable reducing agent was added to initiate growth of this organism at the required low oxidation-reduction potential. Boiling the medium to expel dissolved oxygen was similar in effect to adding ascorbic acid. Although 46 C was established as the optimum growth temperature, a few years later Schmidt, Logan, and Tytell, also at the University of Cincinnati, reverted to 37 C to investigate the degradation of arginine by C perfringens. The lower temperature seems to have been prompted by the universal availability of incubators set at 37 C in microbiological laboratories, and considerably fewer at 46 C. Such was the situation at the University of Kentucky where all incubations for this project were done at 37 C. According to data in Bergey's manual 22 species of Clostridia are capable of good growth at temperatures well above 37 C. Therefore, adjustment of incubation temperature for differential growth purposes would appear to be of little selective value. However, growth at the higher temperature could be expected to have an inhibitory effect on any aerobes that might be present if sterilization of the glucose component, which was done at 100 C instead of 121 C, was not complete.

Production of large inocula: In studying the response of action of enzymes of C perfringens on amino acids it was desirable to have available a source of the combined cell-free enzymes of this organism in as pure a form as possible. A survey of all major suppliers of enzymatic products revealed that a very meager supply of enzymes of this anaerobe were available commercially. Furthermore procurement would be slow and difficult, and the cost prohibitive. It was therefore decided to conduct the research using the washed cell suspensions directly on each amino acid substrate in a buffered solution of the nutrient salts.

Cells for study of production of ammonia from amino acids were at first grown in a manner analogous to the method that Aiken used successfully for her dissertation on deamination by Brucella. She produced the Brucella on trypticase soy agar slants and suspended the cells from each of the slants with 0.6 ml of 0.85 per cent salt solution. The substrates she used were comprised of 0.9 ml of 0.5 per cent solutions of each amino acid buffered with appropriate salts to the desired pH. The inoculum consisted of 0.1 ml of the combined cell suspensions. The turbidity of this suspension was

equivalent to a number 10 MacFarland nephelometric standard or approximately 3×10^9 cells per ml. One tenth of this number was the actual inoculum used.

Trial runs proved that Aiken's procedure had several disadvantages which detracted from its usefulness for study with C perfringens. It was slow and laborious to produce large inocula of the organisms by growing them on agar slants in test tubes. Suspension of the cells in 0.6 ml of saline from many test tubes proved to be a tedious and time consuming process. The numerous tubes used demanded considerable space in anaerobic jars. Due to its high protein content the small amount of medium that dissolved from the surface of the agar as the organisms were put into suspension was sufficient to give a slight positive Nessler reaction. The experience gained in testing the twelve sterile media for ammonia, previously mentioned, proved of value in making this observation. Remedy of this latter objection was, however, possible by washing the cells twice by centrifugation, and resuspending them in sterile saline.

The next experiment was designed to find out from what compounds Clostridium perfringens would liberate ammonia. Until a better method of cell production was decided upon, a modification of Aiken's procedure was tentatively used. A heavy suspension of cells was prepared by growing the organisms on liver veal agar slants. This medium contained the essential nutrients required for good propagation of the anaerobe; and was available for immediate use to maintain progress on the project. The cells were grown in Brewer jars under hydrogen for 18 hours at 37 C. They were suspended in small amounts of physiological salt solution and pooled in a test tube. As expected, Nessler's solution gave a positive reaction on a sample of this suspension due to uptake of soluble matter containing ammonia from the agar slants. Therefore, the cells were washed three times with 0.85 per cent salt solution by centrifugation at 3000 RPM for 15 minutes in a slanted head, and resuspended in a buffered salt medium. With this washing treatment the test for ammonia was negative. In later experiments two such washings were found to be sufficient. The buffered medium in which the cells were resuspended consisted of the six mineral salts listed previously which Boyd, Logan and Tytell determined to be required for optimum growth. This isotonic mineral nutrient buffer was considered to be a much more satisfactory environment for the test organism than 0.85 per cent salt solution. In future experiments the cells were always taken up in this nutrient mineral buffer rather than isotonic salt solution, and also washed in it. After several techniques for obtaining large inocula of the various species from this liquid medium had been tried, the idea of growing the organisms directly in centrifuge tubes was adopted. Pyrex tubes of 90 ml capacity, of the same external dimensions as 100 ml tubes but of thicker glass to withstand the strain of centrifugation, were used in the entire series of tests, after the variables were stabilized.

Amino acids deaminated by *C perfringens*: The next experiment was designed to find out from what compounds *C perfringens* would liberate ammonia. One per cent substrates of the various compounds to be tested for ammonia production were made by inserting 10 mg of each of these substances into a series of small test tubes and adding 1 ml of the nutrient buffer to each of them. In some cases where the solubility of the compound at room temperature was less than one per cent, such as for example 0.5 per cent of 1-aspartic acid, the substrate was saturated with the compound to be tested. The tubes were plugged with cotton and sterilized at 100 C for 30 minutes in free flowing steam in an Arnold type steamer. A steamer instead of an autoclave was used to avoid possible decomposition of the amino acids at temperatures above 100 C. It is realized that complete sterilization of microorganisms is not achieved unless steam under 15 pounds pressure is used for 30 minutes. The compromise temperature decided upon was justified by the advantage gained in the amino acids remaining unaffected during the sterilization process. The detrimental effect of any surviving contaminant was to be overcome by the mass action of the large number of organisms used in the inoculum.

After adjusting the cell suspension to the turbidity of a number 4 standard MacFarland nephelometer tube, a heavy inoculum of 0.3 ml of this suspension was added to each of the substrates. This indicated that a count of approximately 3.3×10^8 organisms was inoculated into each tube. The tubes were incubated under hydrogen in a Brewer jar at 37 C for 18 hours. Tests for ammonia production were done by transferring a drop of the substrate on a spot plate and adding a drop of Nessler's reagent to it. Nessler's solution was prepared according to the modified formulation used by Archibald, namely; 45.5g of mercuric iodide and 34.9g of potassium iodide dissolved in 150 ml of water. To this mixture 112g of potassium hydroxide was added and the whole diluted to a liter with water. Tests were considered positive for ammonia if the spot became an orange color, characteristic of the Nessler reaction. Results obtained are listed below. The numbers following the nitrogen compound indicate the relative intensity of the orange color observed, the higher numbers showing the more positive reactions. All blank tests were negative.

D-Alanine	0	Glycine	0
L-Arginine monohydrochloride	4	L-Histidine monohydrochloride	0
L-Asparagine	3	L-Hydroxyproline	0
L-Aspartic Acid	0	DL-Isoleucine	0
DL-Citrulline	2	L-Leusine	0
L-Cystine	1	DL-Lysine monohydrochloride	0
L-Cysteine hydrochloride	0	DL-Methionine	0
3, 4 DL-Dihydroxyphenylalanine	0	L-Ornithine	0
L-Glutamic Acid	0	L-Phenylalanine	0
L-Glutamine	6	L-Proline	0

Pyridoxamine hydrochloride	0	Valine	0
Pyridoxine hydrochloride	1	Xanthine	0
DL-Serine	3	Control Nr 1, buffer	0
DL-Threonine	5	Control Nr 2, cell suspension	0
DL-Tryptophane	0	Control Nr 3, buffer and cell suspension	0
L-Tryptophane	0		
L-Tyrosine	0		

Some of these compounds were poorly dissolved, but of the ten that gave positive results only cystine went into solution with difficulty. The results obtained were consistent with those reported by Schmidt, Logan and Tytell for the three compounds considered in their publication. C perfringens degrades arginine to citrulline and ammonia; and citrulline to orithine and ammonia. The ornithine, being an end product in the reaction, is not further hydrolyzed. As expected, ammonia was obtained from arginine and citrulline, but not from ornithine, which was reassuring. Woods and Trim in their initial work did not achieve a breakdown of citrulline with this organism. However, the nutritional conditions they used for the organism were not as optimal as those developed later.

Development of procedures using other Clostridia: In view of the satisfactory positive results obtained with ten of the 31 nitrogen compounds tested, the five amino acids that gave the most positive results were selected for further experimentation.

These were 1-arginine monohydrochloride, 1-asparagine, 1-glutamine, dl-serine, and dl-threonine. To find out what the reactions with other Clostridia on these five amino acids would be, a series of experiments was planned using a few species at a time until the entire collection was tested. It was hoped that the reactions for each species would be sufficiently different from the others so that the results, if not fully characteristic for each member of the genus, would at least turn out to be so for C perfringens.

Numerous experiments were tried with various strains of Clostridia acting on the selected amino acids in order to establish satisfactory conditions for conducting the overall series. The objective of these trials was to stabilize the variables so that the investigation could be done uniformly on all of the species available. Otherwise the results could not be interpreted conclusively. Early in the study it was necessary to find a way of producing cells for large inocula that was more efficient than growing them on agar slants. A medium was needed in which the cells could grow abundantly and from which they could be harvested readily. A liquid medium would of course be the answer; but it was important that it be free of suspended matter. Any suspended matter would be deposited on centrifugation and would interfere

with recovery of cells. Cells were required to be free of adhering nitrogen compounds contained in the medium. Several good growth media for Clostridia consist of infusions of liver, or meat, or brain, or heart, or combinations thereof. These cooked sterile tissues contain reducing substances and nutrients ideally suited for anaerobic growth conditions. Unfortunately the tissues are suspended or settled in these fluid infusions and it is not practical to separate bacterial cells from the deposited particles. Consequently none of these classical media was used. Instead a soluble medium was patterned which included all of the nutrients needed for optimum growth of Clostridia. It must be borne in mind that some media which are clear when first dissolved become turbid upon sterilization with heat. Brain heart infusion medium for example is clear before sterilization, but develops a sediment after autoclaving. The medium developed for this project was practically clear upon preparation but became clouded with a fine flocculation after it was sterilized. This was remedied as described later.

Growth medium for Clostridia: The formulation of the growth medium for Clostridia included the mineral salts previously listed excepting manganese which later workers found was not required. A low concentration of glucose and three rich sources of proteins rather than the chemically defined nitrogenous compounds required by C perfringens completed the medium. Its composition is given below.

Component	Per Cent	Grams per Liter
Yeast extract	0.5	5.0
Trypticase	1.5	15.0
Phytone	0.5	5.0
Glucose	0.1	1.0
K_2HPO_4	0.85	8.5
KH_2PO_4	0.15	1.5
NaCl	0.1	1.0
$MgSO_4 \cdot 7H_2O$	0.025	0.25
$FeSO_4 \cdot 7H_2O$	0.00125	0.0125

Glucose is sterilized separately and added to the other sterile components. Otherwise it would cause browning which may be toxic. Although phytone, a soya meal hydrolysate, contains an appreciable amount of carbohydrate, a low concentration of glucose was retained as a fast starter energy source to initiate growth quickly. The concentration of sodium chloride was also reduced, since little of it is required by the Clostridia. Based on the work of Shankar and Bard the manganese salt was excluded. These authors made an extensive study of the effect of metallic ions on C perfringens and proved that manganese is not required for optimum growth. They also proved that

calcium was an essential requirement. Calcium was not listed among the nutrients required for optimum growth. However, this element is evidently present in the protein constituents of the medium, especially in the casein hydrolysate.

Ster..ization of media components and cultivation of Clostridia: In reality only the first three components of this medium remain clear after autoclaving at 15 pounds pressure for 30 minutes. The minerals alone, when dissolved, will form a small amount of precipitate because the iron and magnesium phosphates that are formed are insoluble. Phosphates also form a small flocculent suspension with some of the protein nutrients. Consequently in practice this medium was made clear by first sterilizing it in the autoclave, letting the flocculent materials settle, decanting the supernate into 90 ml centrifuge tubes containing the sterile glucose component, and discarding the sediment. Thus prepared the 90 ml centrifuge tubes were charged with 60 ml of the complete medium and were ready for inoculation for production of large quantities of cells of the various species. Inoculation was accomplished from stock cultures maintained as stated below. It proved to be very convenient to produce the organisms in quantity directly in these large centrifuge tubes. Luxuriant growth was obtained after 18 hours incubation at 37 C. Anaerobiosis was accomplished by flushing the Brewer jar with hydrogen, igniting the residual oxygen, then partially evacuating the hydrogen. Experience proved that evacuation was desirable in order to maintain a tight seal to compensate for gas production by the anaerobes and for expansion of the gases due to heat of incubation. When the small tubes containing the amino acid substrates and heavy inocula were prepared for anaerobic incubation the evacuation step was omitted because of the small volume of gas produced. Evacuation could have caused some loss of the ammonia produced if the tubes had been under reduced pressure.

Stock cultures of the 22 available strains of Clostridia that were used to inoculate the growth medium in the large centrifuge tubes were maintained by frequent transfer to brain-liver-heart semisolid medium. These transfers were incubated anaerobically at 37 C for 48 hours and stored in the refrigerator at 5 C. Cells were harvested by centrifugation at 3000 RPM for 15 minutes in a heavy duty centrifuge capable of spinning four of the 90 ml tubes at a time. The organisms were washed twice by centrifugation and resuspension in a nutrient buffer medium containing all of the components of the growth medium listed above except the first three substances. After the second washing the supernate was checked for absence of ammonia with Nessler solution.

Conditions for testing amino acid substrates: The turbidity of the cell suspension was adjusted to correspond to a number 4 MacFarland nephelometer tube.

Then 0.3 ml of each organism was inoculated into substrates containing one per cent of the amino acid under test which was dissolved in the same nutrient buffer as used to wash the harvested cells. It was important to introduce these large inocula promptly and place the inoculated tubes into anaerobic jars immediately. Otherwise during exposure to high oxygen tension the viability of the cells would be grossly impaired. Overnight storage of the cells in a refrigerator for example caused over 90 per cent loss of viability, probably due to oxygen tension and lack of nourishment. For each organism and each amino acid reaction studied, three controls were incubated simultaneously with the basic experiments. These consisted of the inoculum with buffer but no amino acid; the amino acid with buffer but no inoculum; and a test of the viability of the inoculum. The latter was done by streaking a loop full of the centrifuged cells on liver veal agar slants and observing the growth after anaerobic incubation at 37 C for 18 hours. Sterilization of the small tubes containing the buffered amino acid substrates was done at 100 C in free flowing steam in an Arnold steamer. The inoculated substrates were incubated at 37 C for 18 hours in a hydrogen atmosphere in a Brewer jar which was not evacuated in order to prevent loss of ammonia.

Substances interfering with Nessler test for ammonia: Efforts to improve the environment of the cells during the washing process and while adjusting their turbidity and inoculating the substrates were fraught with considerable difficulty. Boyd, Logan, and Tytell used ascorbic acid as a reducing agent to effect anaerobiosis in the optimum medium developed for C perfringens. Ascorbic acid was tried as recommended. It was added in a concentration of 250 mg per liter to the nutrient buffer just before washing the centrifuged cells. It also was used in preparing the amino acid substrates. However, after recovering the washed cells from 8 species of Clostridia and inoculating the 5 amino acid substrates and controls with the organisms, all 54 tests after incubation gave a uniformly positive Nessler reaction.

It quickly became apparent that ascorbic acid reacts with this reagent to give a false positive Nessler test. When other reducing agents such as cysteine or sodium thioglycollate were substituted for ascorbic acid, false positive Nessler tests were similarly obtained. The reactions were not as intense as with ascorbic acid; but even in lesser degree they were sufficiently strong to mask interpretation of true positive tests.

Besides reducing agents other materials in the substrate interfered with interpretation of the Nessler reaction. Ferrous iron and magnesium reacted with the reagent forming precipitates. These presumably were hydroxides formed from the alkali in the reagent. Furthermore the mercuric ion of this reagent would function as a protein coagulant. Nessler solution was also influenced by the wetting agent Tween 80. A small amount of the latter had been

used in the nutrient buffer while washing the cells of certain species. The objective of the Tween 80 was to disperse clumps of the bacteria caused by the cells packing during centrifugation. This wetting agent was especially needed to prepare uniform suspensions of C sordelli because of considerable clumping of the cells. Tween 80 was also required to disperse C botulinum Type C, which became very slimy on sedimentation. However, Nessler solution formed a white precipitate with this wetting agent. Nessler reagent would be adequate if the ammonia produced could be distilled or separated from the interfering substances present in the substrate prior to applying the test. But the extra work that would be involved in doing this would defeat the purpose of a rapid test.

Trials with other tests for ammonia: Considering the problems presented by the use of Nessler reagent it was decided to explore the possibility of using other methods for detecting ammonia. Thornley in her arginine test for Pseudomonas detected the alkalinity produced by ammonia by means of a pH indicator. Since this classical method was used in as recent a publication as hers, it appeared to have distinct possibilities for use in this project as apparently ammonia would be the only source of alkalinity. Of several standard indicators tried, neutral red seemed to be the most useful on account of its solubility in water, its narrow range between acid and alkaline, and its color change near the point of neutrality. Various concentrations and amounts of this indicator were applied in different ways. Tests with known concentrations of ammonia proved that appreciable amounts of it were needed to be detectable by neutral red. For example, amounts of ammonia that would readily turn phenolphthalein red in water would not change the neutral red from red to yellow in the buffer. Some species of Clostridia acting on arginine did, however, produce sufficient ammonia to affect the indicator in the presence of buffer. But positive tests could not be observed with certainty on the other amino acids because the color change with incremental amounts of ammonia was too gradual and therefore insignificant.

In general, the experiments with neutral red revealed that the buffering action of the mineral substrate was too effective for the small amount of ammonia produced to raise the pH noticeably. Consequently the indicator was insensitive to the slight alkalinity usually manifested in positive reactions. Curtailing the carbohydrate content of the medium to reduce metabolic acidity was not helpful either. If the buffer had been eliminated to increase the sensitivity of the indicator, the favorable environment furnished by the substrate would have been sacrificed. Therefore, a method not affected by buffering action had to be found for detecting ammonia produced in the substrate.

Four other methods for detecting small amounts of ammonia were considered. One of these on which Parnas had published numerous papers depended

on distillation of the ammonia, and consequently required relatively large samples. It was therefore not suited for the purpose of this project. Similarly the procedure of Haussler and Hajdu was not sensitive enough for the micro quantities produced in bacterial cultures, as its mean deviation was plus or minus one milligram of ammonia per hundred grams of sample. Another method, devised by Thomas, was specific for ammonia in weak solutions; was supposed to remain unaffected by media components; and could be applied to bacterial cultures modified as follows:

Dilute 0.2 ml of culture with 8 ml of water. Add 1 ml of 4 per cent aqueous phenol and 1 ml of sodium hypochlorite of 1 per cent available chlorine. A blue color develops to indicate liberated ammonia. If the test solution is amber a greenish blue or green color will be obtained as a positive index of the presence of ammonia.

Selection of the Hansen test for ammonia: The chemistry of the Thomas test was improved by Hansen. He substituted hypobromite for the hypochlorite because it did not vary in strength, thus permitting preparation of a stable reagent. Thymol instead of phenol made it possible to extract the blue color with a small volume of ether, resulting in a beautiful red-violet solution which floated to the top of the aqueous layer. The extracted color was more intense in the ether layer than it had been in aqueous solution. Therefore, his modification was more sensitive than that of Thomas. Hansen's procedure as adapted for use in this project is given below:

Hansen Test for Ammonia

Reagents

A 100 ml 2 N NaOH
B 2 g thymol plus 10 ml 2 N NaOH plus 90 ml water
C 20.6 ml 2 N NaOH plus 79.4 ml saturated bromine water

Procedure

To one ml of test solution add 0.2 ml of reagent A to neutralize any acidity and make the solution slightly alkaline. Shake. Add 0.2 ml of reagent B and mix again. Then add 0.2 ml of reagent C and shake. Let stand to develop the blue color. After 20 minutes add 0.5 ml of ether and mix gently by inversion several times to avoid formation of an emulsion. Let stand until the ether rises.

Observation

Positive tests will develop a blue or green color before the addition of ether. The substance producing the color is soluble in ether and changes to

red-violet as the ether accumulates on the surface of the aqueous solution.

Controls

To test the sensitivity of Hansen's method, and as a positive control on the reagents, serial dilutions of ammonium chloride from 1:100 to 1:100,000 are incubated along with the cultures each time and tested for ammonia.

Discussion

In general, reagents for detecting ammonia include alkali in order to liberate the ammonia in the event it has combined with an acid in the substrate and formed an ammonium salt. Hansen's method requires that the substrate be neutral or alkaline when the test is applied. If it is not, sufficient alkali is added to increase the pH to 7 or slightly higher. This of course is the function of reagent A. Even though the reagent contains alkali for neutralizing the acidity of the medium, additional alkalinity incorporated in reagent B is required to produce the specific color reaction between thymol and ammonia itself. The reagent should not be depended upon to neutralize an abnormally acid substrate and thereby use up some alkali needed for the test. It is therefore important to check to be sure that the substrate is neutral or slightly alkaline before reagents B and C are added.

Detection of ammonia in bacterial cultures is often taken for granted as being quite a simple and routine matter. But the observations made during this study at the University of Kentucky with different methods for detecting small amounts of ammonia leads the author to suspect that an appreciable number of false positive reactions may have been reported in the literature by analysts who unwittingly performed the test for certain purposes without investigating it.

Of the five methods studied, Hansen's procedure appeared to be the most specific for ammonia and the most practicable. It was therefore adopted and used as the standard test for ammonia throughout the series of reactions of the 22 species of Clostridia on the five amino acid substrates chosen. Nevertheless, it had to be adopted with some misgivings because Aiken reported certain difficulties in interpreting the results of her Brucella deamination studies with the test. There appeared to be some definite disadvantages in using the method. In some cases the solutions became amber or bright orange in color after the addition of the three reagents. Ordinarily such non-blue or non-green colors would have been construed as negative tests. However, when extracted with ether the characteristic red-violet hue appeared. On the other hand, occasionally, the aqueous solution was blue or green as if

positive for ammonia, but failed to show the expected red-violet color in the ether layer. Such tests, therefore, were a matter of arbitrary decision rather than objective evaluation as to which was positive and which negative. This difficulty with the amber color was encountered in our observations of several species of *Clostridia* acting on arginine. An amber color appeared with a few species when the reaction was positive, and with a few when the reaction was negative. This amber color did not occur with any of the other four nitrogen compounds tested. Sufficient time was not available to investigate why the substrates containing arginine showed this distinct amber color in certain cases and not in others.

In general, the disadvantages experienced in using Nessler's reagent were not apparent in applying Hansen's method for detecting ammonia. There was, however, one exception. Tween 80 could not be used to disperse slimy or packed cells in conjunction with Hansen's method because an emulsion of the aqueous and ether layers was formed which did not separate on standing. Consequently, clumped cells had to be dispersed by shaking the suspension as well as possible, and a larger volume of the less turbid inoculum used in order to achieve the desired concentration of cells. Strong reducing agents such as ascorbic acid and sodium thioglycollate also interfered; but they had been eliminated from the medium in studying the problems caused by using Nessler's reagent. Instead, the concentration of glucose in the nutrient buffer was increased from 0.1 to 0.5 per cent. Glucose was the least active of the reducing agents studied for use in maintaining a low oxygen tension while harvesting the cells and processing them for the substrate tests. Nessler's reagent would not tolerate even the 0.1 per cent concentration of glucose used earlier in the nutrient buffer when other reducing agents were being examined. However, Hansen's procedure functioned quite satisfactorily in the presence of 0.5 per cent glucose. The latter concentration was adequate for the reducing conditions needed to prevent loss of viability of the cells during recovery and substrate tests.

As may be gleaned from the observations reported with several methods for detecting ammonia, it is not always possible to avoid some false positive reactions. Occasionally substrates containing compounds that react like ammonia may give pcsitive results that could be misleading. For example Hansen's method also gave positive tests in the presence of certain aliphatic amines or glycine. These reacted like ammonia. This may be explained by the extreme sensitivity or lack of absolute specificity of Hansen reagent. However, it must be said in favor of Hansen reagent that it far outclassed Nessler solution insofar as specificity for ammonia is concerned. It must also be borne in mind that production of traces of ammonia cculd

conceivably be caused by the large mass of living organisms of the inoculum acting on the small proportion of cells that had lost their viability during the recovery and test processes. Very weak reactions of this sort were usually interpreted as being negative. These anomalous experiences with such unusual variants are worth mentioning as a warning that no matter how fundamental a reaction is considered to be, exceptions may be expected to occur. Therefore, the analyst must be aware of such possibilities in order to judge correctly the reactions that may be occurring in spite of these anomalies. The infrequent occurrence of such anomalies should not be construed as limiting the usefulness of the procedure for the purpose intended.

Results: Table 1 shows the results of degradation by *Clostridia* of the five nitrogen compounds selected. Production of ammonia was taken as an index of a positive reaction.

PRODUCTION OF AMMONIA FROM AMINO COMPOUNDS ' Y *Clostridia*

SPECIES	L-Arginine HCl	L-Asparagine	L-Glutamine	DL-Serine	DL-Threonine
C bifermentans	+	-	+	-	+
C botulinum Type A	+	+	+	+	+
C botulinum Type B	+	+	+	+	+
C botulinum Type C	+	+	+	+	+
C capitovale	+	-	+	-	+
C carnis	-	-	-	-	-
C difficile	+	-	+	+	+
C histolyticum	+	+	-	-	-
C novyi Nr 17 A	+	+	-	-	+
C novyi Nr 40 A	+	+	-	-	+
C oedematoides	+	+	+	-	+
C parabotulinum	+	+	+	+	+
C perfringens Nr 1	+	+	+	+	+
C perfringens Nr 2	+	+	+	+	+
C septicum	+	-	-	-	+
C sordelli	-	-	-	-	-
C sporogenes Nr 1	+	-	-	+	+
C sporogenes Nr 2	+	-	-	+	+
C tertium	-	+	+	-	-
C tetani Nr 3	+	+	+	+	+
C tetani Nr 43	+	+	+	+	+
C tetanomorphum	-	+	+	+	-

Conclusion and recommendation: Some of the reactions reported in the table above were done many times and consequently were thoroughly verified. But a majority of these tests was performed only once. This was due to the fact that the experiments were conducted during the process of adapting and developing the techniques and while investigating the biochemical aberrations encountered during the progress of the study. The research was performed intermittently as time became available between lecture sessions and after laboratory course periods. Research accomplished piecemeal as circumstances sometimes dictate is not the most efficient way of undertaking a project. Since all of the reactions reported were not verified, it would be reassuring to repeat the tests shown in the table in a sustained and continuous effort. If the final observations were verified using the improved techniques and stabilized variables uniformly there could be no doubt as to the validity of the results. This should be done before this paper is published. With this thought in mind a few suggestions are offered for extending the work to make the scheme more selective, and for facilitating the procedure to accomplish the work more effectively and more quickly.

To a large extent the table is selective for C perfringens because the results are uniformly positive for all five nitrogen compounds. This eliminates all but three of the other species, namely, the botulinum, parabotulinum, and tetani strains. The latter have certain characteristics which make it possible to differentiate them readily from C perfringens. It would nevertheless be desirable to make the table completely diagnostic. This of course could be done by extending the cross reaction studies to all of the nitrogen compounds tested, and even to several more significant ones that can be made available. It is reasonable to expect that from a broader table a more decisive analytical key could be abstracted than the one presented. More species of Clostridia could also be included. Additional species are in existence but are not as readily available as would seem apparent. Collectors are often slow or reluctant to submit transfers of their rare species when requested. The "rare" species sometimes turns out to possess characteristics that are no different from those of a similar species already contained in the seekers collection. Furthermore the transfer may be in poor condition. Several months time would be required in order to extend and activate the collection of Clostridia already available.

With more nitrogen compounds and a longer list of Clostridia the number of cross reactions to be examined in an extension of this project would be multiplied several fold. The study could be expedited by first preparing and storing in the lyophilized state, cells of all the species available. Meister, Sober, and Tice found that the enzymatic activity of lyophilized cells stored

at 5 C is constant for at least six to eight months. The cells are merely suspended in the proper buffer solution for use in preparing the inoculum of required turbidity. After the test for Clostridium perfringens is finalized, such a supply of lyophilized cells would be a most advantageous asset in any diagnostic laboratory. These would be of tremendous value for test verification purposes.

When the supply of lyophilized cells had been made available a concerted effort could be made on the amino acid study. Efforts could be directed toward shortening the time required for incubating the substrates. Although our substrates were incubated 18 hours, this was done chiefly for overnight convenience. Schmidt, Logan, and Tytell incubated many of their arginine substrates for only 90 minutes. Shortly after an hour's incubation time their ammonia production, and arginine disappearance curves were practically level. All tests for arginine from 3 hour incubation mixtures were negative. Reducing the incubation time to an hour or two not only would expedite the extended research effort, but also would establish more rapid conditions for performing the eventual routine test for Clostridium perfringens. In this respect an idea worth exploring suggests the use of a hypertonic rather than isotonic buffer to urge secretion of a higher concentration of enzymes, and liberation of less diffusible ones. This might be expected in the resulting higher osmotic pressure substrate. If a greater number of enzymes would become available through this expedient still faster results in accomplishing the tests could be expected.

The action of only ten species of Clostridia on individual amino acids has been reported in the literature as reviewed recently by Barker. Five of these species were different from those included in this study. The remainder were the only few considered here that were previously reported upon elsewhere. This is an indication of the low incidence of studies of this nature. Consequently, favorable consideration for seeking more information on this subject by an extension of this study as suggested appears to be justified.

PART II

Report of project

INVESTIGATION OF <u>Clostridium perfringens</u> IN BEEF STORED AT VARIOUS TEMPERATURES

Accomplished during the period

17 July to 31 December 1961 at the

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EFFECTS OF MAINTAINING FOOD POISONING STRAINS OF *Clostridium perfringens* IN BEEF AT DIFFERENT STORAGE TEMPERATURES

Summary: It is not possible to keep *Clostridium perfringens* out of food. There is no way of avoiding it because it is universally present. It has been isolated from fruits, vegetables, dairy products, processed foods, meat, fish, and fowl and is a normal inhabitant of the intestines. The best that can be done is to keep it from multiplying profusely in any one situation. Most food poisoning outbreaks have occurred because food was not properly refrigerated or, in cooking, the food was not sufficiently heated to kill the spores of heat resistant non-hemolytic strains, thus, on cooling, enabling spores to germinate and organisms to multiply to fantastically high numbers. Cooks do not realize that when they heat food to about 55 C, although it appears fairly hot, it is inadequate from the standpoint of killing even vegetative cells. Elizabeth McKillop found *C perfringens* in 40 per cent of cooked food as it reached the table.

To prevent frost from building up on the walls of refrigerators large cuts of hot meat are frequently left to cool at room temperature, sometimes until the next morning. Situations like these are exactly the kind that permit the scant numbers of *C perfringens* that may initially be present to rise in concentration exponentially. While these are extreme examples, it was considered important to investigate the survival and growth of this organism in beef stored under various conditions at several temperatures for different lengths of time. This would give an indication of which conditions to avoid and which to recommend for safe use.

Our study at the Low Temperature Research Station revealed the following. Spores of a highly heat resistant food poisoning strain of *C perfringens* survived 100 C for more than 5 hours while other heat resistant strains lasted gradations of this much time down to less than 5 minutes. Our survival curve for the most heat resistant strain shows that the number of spores was reduced tenfold for every hour of steaming at 100 C. Spores of this heat resistant strain survived more than 6 months storage in frozen meat at minus 5 C and minus 20 C. Vegetative cells survived better at the latter temperature than at minus 5 C. These cells died gradually when stored for 13 days at 1 C, 5 C, 10 C, and 15 C; but at the same time, the spores remained fairly constant. Multiplication was slow at 20 C but increased appreciably at 25 C and was rapid at 37 C. About 3 per cent of the spores germinated without prior heat shock. Therefore the majority failed to germinate in raw meat at any temperature. They did so once the meat had been heated. After heating the meat and allowing it

to cool, almost all of the spores had germinated. Experiments indicated that meat prepared, or kept at a pH higher than 5.8 would probably have a lower minimal growth temperature for C perfringens than at a lower pH.

Introduction: Most outbreaks of Clostridium perfringens food poisoning result from meat products kept warm or left at room temperature for hours before serving. Under these conditions the heat resistant spores of the organism, that are present in the raw meat and survive cooking, germinate and multiply. Reports of this type are frequent in England because public health officials there recognize the problem and take steps to investigate it. On the contrary, reports of food poisoning by C perfringens in the United States are rare because of incomplete analysis for extremely few stool examinations are made which would reveal that a case of food poisoning is indeed caused by C perfringens.

Typical of such cases is the one that occurred in Berkeley, California on 26 September 1959. A 22 pound beef roast was purchased the previous day and refrigerated overnight. The cateress roasted the meat only 2-1/2 hours, from 7:30 a.m. to 10:00 a.m., and removed it from the oven for cooling until noon. Then it was sliced, tied, and left at room temperature until 3:00 p.m. when it was reheated in the oven for only 20 minutes. After another cooling period the meal was served at 6:00 p.m. and the dinner was over at 7:30 p.m. Most of the persons who became sick, some 15 hours later, were afflicted with acute abdominal cramps and diarrhea. Bacteriological analysis revealed the presence of C perfringens in a concentration of 580 million organisms per gram of the leftover meat, which was not put in the refrigerator until midnight.

Conditions prevailing in this case were obviously unsuited for serving a wholesome roast beef dinner. Everything that the cateress did here was wrong. The meat was too large, the cooking was done too soon and too briefly, and the short reheating period came between two slow cooling periods. By slicing the meat contamination was distributed throughout; and by tying the slices together anaerobic conditions were maintained. Thus a generous opportunity for spore germination and cell multiplication was afforded.

Studies on C perfringens under these extreme circumstances was not the purpose of the portion of this Fellowship project that was done at the Low Temperature Research Station in Cambridge. Rather, the objective consisted of an investigation of the growth, sporulation, germination, heat tolerance and recovery of C perfringens under similar, but more fundamental and discriminating conditions. It was desired to learn how these organisms would be affected if they were maintained in raw beef, at several different temperatures for storage periods up to 6 months. No investigations of this nature have been done

on food poisoning strains of *C perfringens* on raw meat, and only very few studies have been accomplished on cooked meat. The latter includes one such study of the multiplication of this organism in stews and reheated sliced meats conducted by Betty Hobbs in 1962. To be safe, she recommended, meat must be stored below 15 C or above 60 C. In heating experiments conducted by Sylvester and Green, *C perfringens* appeared to survive a slow overnight roasting. However, the temperature in the center of the cut reached only 66 C after 9 hours. Consequently, the authors saw no advantage in overnight cooking. With reference to experiments with raw meat, only the incidence of *C perfringens* in different species of animals has been reported. As expected, this incidence runs high.

In this project small cubes of sterile raw beef were inoculated with known amounts of a food poisoning strain of *C perfringens*. These were stored at temperatures ranging from minus 20 C to plus 37 C. Survival and growth of vegetative cells and spores for storage periods up to 6 months and more, at these temperatures, were determined. In the preliminary experiments the several food poisoning strains of *C perfringens* to be investigated were tested for the possible presence of contaminants. Then spores were produced, vegetative cells eliminated, and counts made by several different techniques to be described. After that a series of heat resistance tests was conducted. Finally the storage study in raw meat cubes was undertaken with a few of the tests being done on heated meat cubes. A description of the strains used, procedures applied, and observations made follows:

EXPERIMENTAL

Brief histories of food poisoning strains of *C perfringens* used as stock cultures:

F-2985-50	Nonhemolytic, serological type 1. Isolated from cooked salt beef in an outbreak of food poisoning.
F-1546-52	Nonhemolytic, serological type 10. Isolated from roast meat in an outbreak of food poisoning.
F-1077-61	Nonhemolytic, serological type 4. Isolated from brisket of beef in an outbreak of food poisoning.
F-3278-61	Nonhemolytic, serological type 3. Isolated from reheated steak and kidney pudding in an outbreak of food poisoning.
F-4022-61	Nonhemolytic, untypable by sera 1 to 13. Isolated from steak in an outbreak of food poisoning.

F-4465-61 Nonhemolytic, serological type 3. Isolated from steamed lamb in an outbreak of food poisoning.

F-4947-61 Nonhemolytic, serological type 11. Isolated from beef in an outbreak of food poisoning.

Maintenance of stock cultures: Strain F-2985-50 was used for most of the experiments conducted during this study. Cultures were preserved in one ounce bottles of Robertson cooked meat medium. Regular checks for purity of all cultures were made during the experimental work. The medium was inoculated from a select colony by means of a platinum wire loop or, up to 0.5 ml was transferred from a culture already grown in cooked meat into the same type of medium. Usually two or more bottles were inoculated with the same culture and labeled "STOCK". The inference of such a label is that the culture has not been used after it has been inoculated and incubated and is therefore the equivalent of a prime culture. Rejuvenation of a stock culture that has been stored for some time is effected by subculturing it to fresh cooked meat medium. Such cultures of spore forming anaerobes may be preserved for long periods of time by storing in the dark at room temperature after inoculation and initial incubation for 24 hours at 37 C.

Confirmatory test for Clostridium perfringens: The Nagler lecithinase test distinguishes C perfringens from almost all other Clostridia. This enzyme is present in all types of C perfringens. It causes a visible dense opalescence due to the hydrolysis of the lecithin containing phospholipid in Nagler media. This reaction is inhibited by the antitoxin of lecithinase which is known as the alpha toxin of C perfringens. The Nagler reaction is not entirely specific for C perfringens since a few other species of Clostridia produce opalescense--to a degree--in egg yolk media. C botulinum and C oedematiens give weak reactions compared with C perfringens. These are distinguishable from the reaction with C perfringens because their opalescence is not inhibited by the antitoxin of C perfringens. C bifermentans on the other hand does give a positive Nagler test which is inhibited by C perfringens antitoxin. It is easily distinguished from C perfringens by its inability to ferment lactose. C sordelli, the name used for pathogenic strains of C bifermentans is included in these exceptions to the Nagler test. Nagler test plates are prepared and dried in the incubator. About four small drops of antitoxin of C perfringens are spread over one half of the surface of a Nagler plate and allowed to be absorbed into the medium for several minutes so that no fluid remains on the plate. The inoculum is seeded across the plate in a straight line with a platinum loop from the side containing no antitoxin to the side impregnated with antitoxin. A regular sized Petri plate may be used to test as many as 5 or 6 strains for the Nagler reaction by inoculating the different strains in parallel lines. The plate is incubated

anaerobically at 37 C overnight. Strains of C perfringens will grow across both halves of the plate. The colony streak on the untreated half of the plate will be surrounded by a broad zone of dense opalescence due to the action of the lecithinase on phospholipid. On the antitoxin treated half no zone of opalescence will be present because the lecithinase has been neutralized by the antitoxin.

Sporulation in Ellner medium: Ellner medium was used for inducing spore formation in C perfringens. It has the following composition:

	Per cent
BBL polypeptone	1.0
Yeast extract	0.3
Starch	0.3
$MgSO_4$	0.01
KH_2PO_4	0.15
$Na_2HPO_4 \cdot 7H_2O$	5.0
Adjusted to pH 7.8	

The ingredients are heated in an Arnold type steamer to effect solution. The medium is dispensed in 10 ml volumes in one ounce screw capped bottles and sterilized in the autoclave at 121 C 15 minutes. Prior to inoculation, anaerobiosis is achieved by removing dissolved oxygen by heating the bottled medium at 100 C for 10 minutes, then cooling.

Inoculation is made with 0.5 ml of a 6 to 8 hour culture of C perfringens grown in Robertson cooked meat medium and introduced with a Pasteur pipette into the bottom of the bottle containing Ellner medium.

Cultures are incubated at 37 C for 48 hours, by which time more spores are formed than after 24 hours, although this is not the case for all strains. During the incubation period only a few generations of growth occur prior to the sporulation process. This, too, does not apply to all strains. Variable degrees of sporulation of the several strains studied were obtained, but a sufficient number of spores was produced to conduct the tests. Ellner medium was used because it had been recommended for this purpose. But, surprisingly enough, four of five food poisoning strains that survived 100 C for a longer period than one hour produced spores in Robertson cooked meat medium in less than 6 hours, and the fifth strain did so in less than 20 hours. Furthermore, a considerably higher percentage of sporulation was attained in this medium than in Ellner medium. Therefore, it would have been unnecessary to use Ellner medium had we discovered this fact before most of our sporulation studies were concluded. This was a finding of exceptional interest because most workers had said that it is difficult to induce C

perfringens to sporulate unless the organism is passed through the intestinal tract of an animal, or, unless it is cultured in Ellner medium.

Total viable count in RCM Agar: This procedure, proposed by Ingram and Barnes, saves using an anaerobic jar. It is useful for counting anaerobes in fluid cultures or slurries containing a minimum of solid particles that would not interfere with colony observation. Decimal dilutions of a sample to be counted were made in Hirsch reinforced clostridial medium containing agar cooled to 50 C. One ml of an appropriate range of dilutions was introduced in duplicate test tubes somewhat larger than regular size. The RCM agar was added to a depth of 5 cm and sterile black glass rods about 6 mm diameter were carefully inserted. Colonies would later be visible around these black rods. When the agar was set the medium was sealed with 2 ml more of the RCM agar. The tubes were placed in cans to catch the overflow in case of stormy incubation and were set in a 25 C incubator to control the incubation. If after 18 hours at 25 C the colonies were not large enough to be counted the cultures were set in a 37 C incubator for more rapid growth, and were inspected occasionally throughout the day until a count was possible. After counting, the tubes were replaced in the 25 C incubator overnight for further controlled incubation and verification of the count the following morning. Spores requiring much longer than 24 hours to germinate would not be detected by this method.

Elimination of vegetative cells: Vegetative cells were destroyed by heating a slurry of the sample in a test tube in a water bath held at 85 C 5 minutes. The 5 minutes timing was based on the temperature reaching 85 C as measured in a similar test tube placed parallel to the tube containing the sample. Then the tube was plunged into cold water for a few minutes. Thus the culture was held at 85 C fully 5 minutes.

Spore count in RCM agar: Decimal dilutions of the preheated spore suspension were made to a proper range of concentrations as described above for total viable count using the black rod technique. For the spore count it was found unnecessary to carry the dilutions beyond 1 to 100,000.

Spore count in cooked meat broth: In cases where the numbers of organisms were small, and where slurries contained interfering meat particles, counts were carried out in an array of Robertson cooked meat medium bottles. These were incubated at 37 C for several days in order not to overlook late germinating spores. Either 3 or 5 replicates were made at each of 3 or more decimal dilutions. Where 5 replicates were used the most probable number was calculated from the tables of the Ministry of Health Report published in 1956. Since the greater proportion of these counts was made using 3 replicates, and the probable number taken from the tables of

Demeter, Sauer and Miller, their procedure will be described in some detail. Since bottles containing turbid Robertson cooked meat medium are used in this statistical method, growth or no growth is the criterion for a positive or negative reading. Three sets of triplicate bottles of this medium are inoculated, each set respectively at an appropriate dilution. One ml of the corresponding inoculum is placed in each bottle of medium. Thus 9 bottles are to be observed. The number of positives obtained after 24 hours or more of incubation is recorded for each set of three bottles from the lesser dilution to the greater. Three figures so obtained give an index number. Opposite the index number a probable number is found in the table of these authors, which is reproduced in the next paragraph. This probable number, with appropriate adjustment for dilution, is the spore count. For example, if all 3 bottles are positive in the minus 1 dilution, 2 are positive in minus 2 and 1 in minus 3 the index number is 321. In line with this index number the probable number given in the table is 15.0. This is accepted as the spore count per ml in the minus 1 dilution. The table is useful for low spore counts, as the limit indicated is only 140.

SPORE COUNT

Index Nr	Probable Nr	Index Nr	Probable Nr	Index Nr	Probable Nr
0 0 0	zero	2 0 1	1.4	3 0 2	6.5
0 0 1	0.3	2 0 2	2.0	3 1 0	4.5
0 1 0	0.3	2 1 0	1.5	3 1 1	7.5
0 1 1	0.6	2 1 1	2.0	3 1 2	11.5
0 2 0	0.6	2 1 2	3.0	3 1 3	16.0
1 0 0	0.4	2 2 0	2.0	3 2 0	9.5
1 0 1	0.7	2 2 1	3.0	3 2 1	15.0
1 0 2	1.1	2 2 2	3.5	3 2 2	20.0
1 1 0	0.7	2 2 3	4.0	3 2 3	30.0
1 1 1	1.1	2 3 0	3.0	3 3 0	25.0
1 2 0	1.1	2 3 1	3.5	3 3 1	45.0
1 2 1	1.5	2 3 2	4.0	3 3 2	110.0
1 3 0	1.6	3 0 0	2.5	3 3 3	140.0
2 0 0	0.9	3 0 1	4.0		or more

Spore count by dilution in RCM broth: Spore counts in Ellner medium were determined after heating the culture 5 minutes at 85 C. One ml of the heated spore suspension was transferred to 19 ml of Hirsch RCM medium containing only 0.05 per cent agar as described by Hirsch and Grinstead. Further 20 fold serial dilutions were made from this into 19 ml lots of the medium. By using 20 fold dilutions instead of the usual 10 ml series a greater depth in the one ounce bottles afforded a greater degree of

anaerobiosis. Furthermore a wide range of dilutions is achieved with relatively few bottles. In this procedure dilutions advance rapidly in multiples of 20 from 1:20 to 1:400 to 1:8000 to 1:160,000 and so on. However, the estimated number of spores is not precise due to the broadness of the end point. The dilution bottles were incubated 24 hours or longer and the spore count was taken to be greater than the number indicated by the greatest dilution.

Heat resistance test for spores: C perfringens strains that were investigated for their heat resistance were produced in Ellner medium. A few ml of the sporulated medium was heated 5 minutes at 85 C to destroy vegetative cells. The initial spore count was estimated by the 20 fold dilution method described above. A 0.1 ml inoculum of this spore suspension was introduced into pyrex ampoule tubes about 9 mm outside diameter and 5 inches long containing cooked meat granules reconstituted in skimmed milk to a depth of 50 mm. Skimmed milk was added according to the recommendation of Crossley to improve the nutritional value of the medium and to aid in detection of growth, since C perfringens ferments lactose readily. Originally bromcresol purple at a concentration of 0.001 per cent was also added as a useful indicator of fermentation. But, it was slightly inhibitory for growth of some of the strains, therefore its use was discontinued. After inoculation the tubes were sealed in an oxygen flame. Then the tubes were immersed in a boiling water bath and subjected to heating at 100 C for intervals ranging from 5 minutes to 6 hours. After heating, the tubes were plunged into cold water and then incubated at 37 C for periods up to 7 days until it was certain that no germination would occur in the negative ampoules. Because the duration of the longer storage studies exceeded my period of assignment at the Low Temperature Research Station, I am grateful to Dr. Ella Barnes for completing these experiments and for other supplementary tests that could not conveniently be done while I was stationed there.

Preparation and Sterilization of meat blocks: A cubical piece of raw lean beef weighing several pounds was superficially sterilized to achieve as aseptic a condition as possible in the following manner. Each side was wet and flamed with alcohol until the surface of the meat was brown as in grilling. Then the outer layer of each burned side was cut off sufficiently to expose the raw meat. In this process sterile trays and instruments were used. The meat thus prepared was cut into about 75 cubes each weighing 15 to 25 grams by estimate of a side about one inch in length. This size was convenient for later handling in 30 ml beakers. These meat cubes were inserted into sterile transparent plastic bags and sealed individually one from another. The meat cubes thus sealed were stored temporarily at minus 20 C until such time as they could be sterilized by irradiation.

Sterilization was accomplished at the Harwell Atomic Energy Establishment spent fuel rod source, Harwell, England by delivery of a total dose of 5 megarads of irradiation, predominantly of gamma rays, at a rate of 1.2 to 1.7 megarads per hour. After that, the cubes were stored at 1 C and drawn upon periodically for inoculation and survival studies over a period of several months until used.

Inoculation of meat blocks: An 18 hour broth culture of *C perfringens* strain number F-2985-50, the most consistently heat resistant food poisoning strain available, was used to inoculate the meat cubes. It was grown in Robertson cooked meat medium for either 24 or 48 hours and then diluted in RCM broth to give the required inoculum. The culture was further diluted to 1:100 with Hartley (p 192 of Mackie & McCartney by Cruickshank) digest broth containing 0.1 per cent sodium thioglychollate and adjusted to pH 5.8 to avoid altering the pH of the meat. A 0.2 ml inoculum was introduced into the center of each meat cube with a 1 ml capacity hypodermic syringe having a small bore needle. The 1:100 dilution inoculum was used on meat blocks number 1 through 45. On meat cubes 46 through 66 a similar culture, but diluted 1:10, was used and the volume introduced was also 0.2 ml. In the inoculation procedure a sterile 30 ml beaker was weighed and the meat cube was transferred from the plastic container into the beaker and reweighed to obtain the weight of the meat. Then the 0.2 ml inoculum was introduced and the beaker covered with aluminum foil and sealed with adhesive tape to achieve eventual anaerobiosis. Thus inoculated, the meat cubes were distributed to various storage temperature chambers for recovery studies.

Heat treatment of meat cubes: Several of the meat cubes which had been stored at 5 C and 10 C were treated with a measured amount of heating to simulate a type of cooking considered to be incomplete for destruction of heat resistant spores. The samples were kept in their beakers and placed in a water bath held at 70 C. A thermocouple was attached to a galvanometer and connected to one of the meat cubes for temperature control. The time required for the center of the cube to reach 70 C was 15 minutes. The meat was kept at 70 C for 30 minutes longer after which an initial count was made on one of the meat cubes and the remainder stored at 37 C.

Bacteriological examination of meat blocks: Total viable count and spore count was determined on each meat cube after a suitable storage time at a given temperature. After removing the aluminum foil cover the meat was comminuted in the beaker with sterile scissors while being held with sterile forceps. Then the meat was transferred to a sterile Atomix, and the scissors, forceps, and beaker were rinsed with 45 ml of maintenance medium consisting of 0.1 per cent peptone and 0.5 per cent NaCl. The mixture was blended in the Atomix without allowing heat to develop by blending first at a low speed for 45 seconds then in high speed 45 seconds.

In this connection Louis DeSpain Smith, in the symposium conducted by Noyes in Chicago, warns against homogenization of specimens containing C perfringens due to appreciable loss from oxygenation. He suggests grinding in a sterile mortar containing sand and saline. The impracticability of macerating these firm raw meat cubes to liberate the organisms for counting was of sufficient importance to preclude using a mortar. To reduce oxygenation to a minimum this blending was done as quickly as possible. The uniformity of this procedure would affect all specimens equally.

The weight of the meat cube plus 45 grams of maintenance medium was taken into account in the calculation required to obtain total viable count and spore count per meat cube. These values represented the uncorrected count obtained for 1 ml of the slurry prepared in the Atomix. Total viable count in RCM agar, and spore count in both RCM agar and in cooked meat were performed as previously stated under the separate headings discussing these topics.

Heat tolerance of C perfringens spores: Before studying the behavior of the vegetative cells and spores of C perfringens in meat the heat resistance of the 7 strains isolated in outbreaks of food poisoning described previously was tested. Spores were obtained from 6 of these strains by culturing in Ellner medium. The strain which could not be induced to form spores was F-1077-61.

To test heat resistance, ampoules containing a cooked meat and milk medium mentioned above were used. The results obtained, using spores which had been in Ellner medium for no longer than 3 days, are given in Table 1. It was found that if the spores were left in this medium much longer they lost some of their heat resistance. For example strain F-4022-61 which usually survived 2 hours at 100 C survived only 30 minutes after it had been in Ellner medium for 48 days. The inoculum of spores tested was the same in each case, and was estimated by dilution in the 20 fold series counting method described above. Five of the food poisoning strains survived heating at 100 C for more than 1 hour. One strain, F-4465-61, survived only 15 minutes. The most heat resistant strain, F-2985-50, when produced in Ellner medium survived 2 hours at 100 C. When spores of this strain were produced in meat broth they survived 4 hours at 100 C. For comparison 5 other strains of C perfringens which did not originate from food poisoning outbreaks were tested for heat resistance. It was very difficult to obtain many spores of these strains in Ellner medium. Furthermore when tested for heat resistance they failed to survive 100 C for 5 minutes.

Storage of inoculated meat cubes at temperatures below freezing: Because of its high heat resistance and its ability to form spores readily in

cooked meat as noted by Collee, Knowlden, and Hobbs, strain F-2985-50 was selected for this storage study in beef. The number of spores formed in Robertson cooked meat broth after 24 hours incubation at 37 C was about 5,000,000 per ml while the total count was 64,000,000 per ml of broth. Small cubes of meat were prepared as described previously. Since large numbers of cubes were required they were sterilized by irradiation as it is very difficult to obtain aseptic blocks by any simpler sterilization procedure without materially altering the structure of the beef. To determine the effect of freezing and subsequent storage at -5C and -20 C, small meat cubes, which had a pH of 5.7, were sealed in plastic bags and were inoculated with about 100,000 organisms including 10,000 spores. The inoculations were achieved using a hypodermic syringe which was pierced through the plastic bag into the center of the cube. The cubes were frozen in a blast freezer for 3 hours. Some were stored at -5 C and some at -20 C and tested after 3, 5, 8, 12 and 26 weeks. The results in Table 2 show that blast freezing reduced the total count about tenfold while the spore count dropped by about one third. Very little destruction of vegetative cells occurred at -20 C after 5 weeks but two thirds of the spores were gone. At -5 C the majority of the vegetative cells had disappeared after 3 weeks and eventually their count was down to 210. Yet 1600 of the spores survived for 26 weeks at -5 C. Only a small proportion of the spores germinated without prior heat shock. While there was some destruction of spores at -5 C, the majority of spores survived at -20 C for the entire 26 week period of this study.

Storage of inoculated meat cubes at temperatures above freezing: Sterile meat cubes having a pH of 5.7 were removed from their plastic bags and transferred into small beakers so that there was little free space. The cubes were then inoculated and the beakers covered with sterile aluminum foil sealed around the rim with friction tape. The residual oxygen in the beaker would be removed by the meat and an anaerobic pack thus obtained. The beakers containing the inoculated meat were held at temperatures ranging from 1 C to 37 C and the numbers of vegetative cells and spores determined. The results given in Table 3 show that there was no multiplication and only moderate destruction of vegetative cells when the meat was stored at either 1 C, 5 C, 10 C, or 15 C. At 20 C there was slow growth of the vegetative cells but no appreciable change in the spore count. At 37 C the vegetative cells multiplied rapidly but the spore count remained about the same. At the intermediate temperatures the spore count decreased slightly but no germination occurred at 37 C and no growth had taken place at 15 C. A similar meat cube which had been stored at 15 C for 11 months showed a count of 30 organisms. Microscopic examination revealed that no multiplication of the cells had occurred. Viable spores dropped in count from 7900 initially to 575 during this period. At 15 C our select strain of C perfringens could not grow in meat cubes which had a pH of 5.8, nor in

RCM broth. But, in Robertson cooked meat medium, at pH 7.2, slow growth was observed. With the thought that this difference was caused by medium and pH an experiment was conducted to test the relationship of minimal growth temperature to pH, adjusting the RCM broth to pH 5.8. RCM broth at pH 5.8 and pH 7.2 was inoculated in triplicate with 0.025 ml of an overnight culture in Robertson cooked meat broth. These cultures were stored at 15 C, 20 C, 25 C, 30 C, and 37 C. Results are shown in Table 4. At 15 C no growth occurred at either pH, but at 20 C growth was faster at the higher pH, requiring 2 days at pH 7.2 compared with 3 days at pH 5.8. In another experiment growth was observed in 4 days at 20 C at pH 7.2, but none after 28 days at pH 5.8 at this temperature. Low pH is, therefore, an important factor in RCM broth at 20 C. Inoculated meat cubes stored at 20 C responded similarly.

Germination of spores in meat cubes: Since no spore germination occurred in the meat cubes at any of the storage temperatures, the following factors were considered as the possible cause:

1. The need for heat activation as a prerequisite for germination.
2. The limiting pH for germination might be higher than for growth.
3. Inhibitors might have been present in the raw meat.

To investigate these considerations meat cubes inoculated with vegetative cells and spores were heated to 70 C and held at that temperature 30 minutes. The results given in Table 5 show that as a result of the heat treatment the vegetative cells were destroyed and the spores were enabled to germinate. After 2 days at 37 C the heated meat cube had practically no spores remaining but contained almost 4 billion vegetative cells.

Heating the meat cubes at 70 C increased the pH from 5.8 to 6.0. In order to confirm that spore germination and growth was not due to this change in pH, spore suspensions were prepared by two methods. One was the usual heat treatment of the broth culture at 85 C for 5 minutes to eliminate the vegetative cells. In the other these cells were destroyed by the lysozyme treatment of Brown, Ordal, and Halvorson. These two spore suspensions were inoculated into raw meat cubes and into meat cubes preheated at 70 C for 30 minutes. The results given in Table 6 show that almost all the heat activated spores germinated and multiplied in both raw and cooked meat stored at 37 C. Only a small proportion of the lysozyme prepared spores could germinate in either sample. With the heated spores in raw meat, a result similar to that at 37 C was obtained at 20 C and 25 C. After 7 days at 20 C the count on the unheated sample was 61 million organisms per meat cube while the spore count was near zero. After 3 days at 25 C the count was more than twice as great, and again the spore count was negligible. It was evident that the heat treatment given the spores prior to inoculation into the meat cubes had enabled them to germinate in both the raw and cooked meat. There was no evidence that any new sporulation occurred in

the meat cubes after the original spores had germinated. This is evident in Table 7 showing a steady drop in the spore count to almost nil in 7 days at 20 C with no gain from the 7th day to the 12th day.

Loss of heat tolerance of spores in meat cubes: Meat cubes inoculated with vegetative cells and spores, when heated at 70 C for 30 minutes, then cooled, and, after macerating, heated to 85 C for 5 minutes yielded a very low count. It must be borne in mind that a germinated spore is, by definition, one which has lost its heat resistance, but has not yet grown out into a vegetative cell. Thus, the spores had germinated during the cooling period and the ensuing vegetative cells were destroyed in the 5 minute reheating period, for, few heat resistant spores remained in the residue. To verify this observation meat cubes were inoculated with cells and spores from a 48 hour culture in Robertson cooked meat broth. One meat cube was sampled immediately, the others were held at 70 C in a water bath for 30 minutes, the temperature being controlled by a thermocouple inserted in one of the cubes. After the heating period, the meat cubes were allowed to cool prior to sampling. One meat cube was sampled and total organisms and spores determined after 15 minutes when its temperature dropped to 32 C; another after 37 minutes when its temperature was 18 C; and a third after 2 hours and 50 minutes at 10 C. When two more meat cubes dropped to 20 C one was held at that temperature for 5 hours and the other for 23 hours before counting. Similarly two more were held at 15 C and sampled after four and 25 hours; and three additional cubes that were cooled to 1 C were tested after 3-1/4, 25, and 144 hours. The counts given in Table 8, showed that the total organisms approximated the initial spore count on the raw meat. Counts on the heated macerates were very low. Apparently, by the time the heated meat had cooled the majority of spores had germinated, as indicated by loss of heat resistance. Little change occurred during further storage at 15 C and 1 C.

This experiment was repeated, inoculating RCM broth with 100,000 spores, heating 30 minutes at 70 C and cooling to 15 C as with the meat cubes and holding samples at 15 C for 3 hours and 24 hours, and at 1 C for 24 hours. Before counting, any germinated cells would be destroyed, for the broths were heated at 85 C for 5 minutes. In this case only a small proportion of the spores had lost their heat resistance, although this is a medium in which the cells grew well. When spores were heat activated and cooled in the presumably spent broth from Robertson cooked meat culture, all spores retained their heat resistance. Under these circumstances the spores were not under conditions where they seemed likely to germinate.

Survival of spores in meat held at 100 C: All previous tests on the heat resistance of spores of *C perfringens* F-2985-50 had been carried out in cooked meat broth having a pH of 7.2. It appeared possible that when

heated in raw beef, which generally has a pH between 5.4 and 5.8, the spores would be less heat resistant. To verify this hypothesis a number of meat cubes were inoculated as previously with 0.2 ml of broth culture which had been diluted with an equal volume of 0.1 per cent thioglychollate digest broth having a pH of 5.8. After inoculation, the meat cubes were held for 2 days at 5 C before carrying out the test. The observations above suggested that this would not affect the situation materially. The cubes were then placed in a small steamer containing cold water and the temperature raised to 100 C. The time required for the center of the meat cube to reach 100 C was 40 minutes. This measurement was accomplished with the aid of a thermocouple fixed within one of the meat cubes. The meat cubes were then sampled at intervals from zero to 6 hours. The number of spores was determined by the dilution counting method taking 5 replicates at each dilution in Robertson cooked meat broth. The last meat cubes were divided between 10 bottles of medium to determine whether any viable spores remained. The results in Figure 1 show that 5 hours and 40 minutes after the meat had reached 100 C some spores still survived. After 6 hours at 100 C no viable spores remained.

Discussion and conclusions: Spores of *C perfringens* survive in frozen meat almost completely at minus 5 C and minus 20 C. However, a high proportion of the vegetative cells are destroyed during storage under these conditions, but less so at the colder temperature. The slow rate of destruction of the vegetative cells in meat stored at 1 C, 10 C, and 15 C, and the stability of the spores at these temperatures indicates that any practical freezing procedure could not be expected to destroy these organisms entirely. Consequently, a high proportion of these organisms would survive for the normal cold storage life of the meat.

With meat at pH 5.8, multiplication occurs at room temperature, starting slowly at 20 C and increasing rapidly at higher temperatures of incubation range. In RCM broth, growth at 20 C was variable at pH 5.8, but rapid at pH 7.2. Meat at the lower pH would, apparently, be less susceptible to spoilage than at the higher one. Heat resistance of spores apparently is not a function of pH, for, spores survived equally as well at 100 C in meat cubes at pH 5.8 as they did in meat at pH 7.2. The death rate of the spores was tenfold for every hour of heating at 100 C, and survival of the longest duration was 5 hours and 40 minutes indicating a heat resistance similar to that of *C botulinum*. Such a high heat resistance would practically assure the presence of viable spores in cooked meat as it reaches the table, unless it were prepared in a pressure cooker.

Less than 3 per cent of the spores of strain F-2985-50 germinated without heat activation. This was evident in almost complete germination in

meat stored at incubation temperature. This proportion is much smaller than is usually the case with aerobic sporing bacilli. Thus, the number of C perfringens spores likely to grow after heat activation is increased by a factor of nearly 100. Consequently, the probability of food poisoning is greatly increased if the spores are present in meat before cooking. After the spores had been heat activated in the meat, practically all of them germinated. Consequently, few or no spores remained to demonstrate heat resistance. By contrast, in vitro, germination occurred with only 75 per cent of the spores in RCM broth. Sporulation of the germinated cells was not achieved under any of the storage conditions in meat prevailing in these experiments.

Although this study was conducted only with beef, it is reasonable to assume that similar results would be obtained with flesh of other species since heat resistant strains of C perfringens are also prevalent in veal, pork, lamb, and fowl.

Recommendation: This study could be enhanced with a more extensive investigation of the survival of C perfringens, and its sporulation, germination and recovery from meat maintained under the different storage and cooking conditions encountered in practical usage. It would be of interest to find out whether or not vegetative cells of C perfringens will sporulate in raw meat at any of the temperatures used in this study and the conditions and effect of pH on sporulation in raw meat. In order to improve progress in these studies it is necessary to find a method of growing more abundant quantities of spores than is possible with Ellner medium. In ordinary media, particularly media that contain carbohydrates, C perfringens does not sporulate, or sporulates very poorly; and, the spores are generally not heat resistant. While Ellner medium may possess some merit for sporulation of C perfringens, many strains do not respond to it; and, some that do respond yield spores that possess characteristics which are different from those of the original. Up to 100,000 spores per ml are needed for ready microscopic observation. Dr. Collee* obtains a higher yield of spores--up to 250,000 per ml--by removing the supernate of a 4 to 6 hour culture from cooked meat broth and replacing it with Ellner medium. This, in turn, is removed with its contained growth after 13 hours at 37 C and reincubated separately from the cooked meat particles for an additional 23 hours. Our efforts to produce more spores in this manner were unsuccessful. It is evident that so much time is consumed in obtaining sufficient spores that slow progress is made with basic experiments.

*Private communication 4 September 1961

The effects of commercial methods of freezing raw meat on the sporulation of C perfringens should be determined. Similarly the effect of heating at sub-lethal temperatures on the sporulation of this organism in meat should be further investigated.

With an abundance of spores we could conduct experiments freely to obtain measurements for their heat tolerance at temperatures both above and below boiling. A heat resistance study of C perfringens at temperatures beyond 100 C should be conducted in order to realize the conditions of factory canning and pressure cooking that would be required to kill the most heat resistant strains. A clue as to the minimum time and temperature food should be cooked to be safe could be obtained with more information on the germination lag of spores of C perfringens following various amounts of heating. Also, more growth curves would be possible starting with pure spore inocula. Serological work with spores would be interesting, too. C perfringens contains the heterophile Forssman antigen. In this connection it would be of value to know the specificity of the agglutination reactions of the antibodies of this antigen and of the spore antigens of the various strains. Much has been written on the sporulation of aerobic species, but there is little to be found in this regard on the anaerobes. Consequently, the undertaking of additional studies to obtain answers to these, and similar questions, is recommended.

Figure 1. Survival of C perfringens spores in meat steamed at 100 C

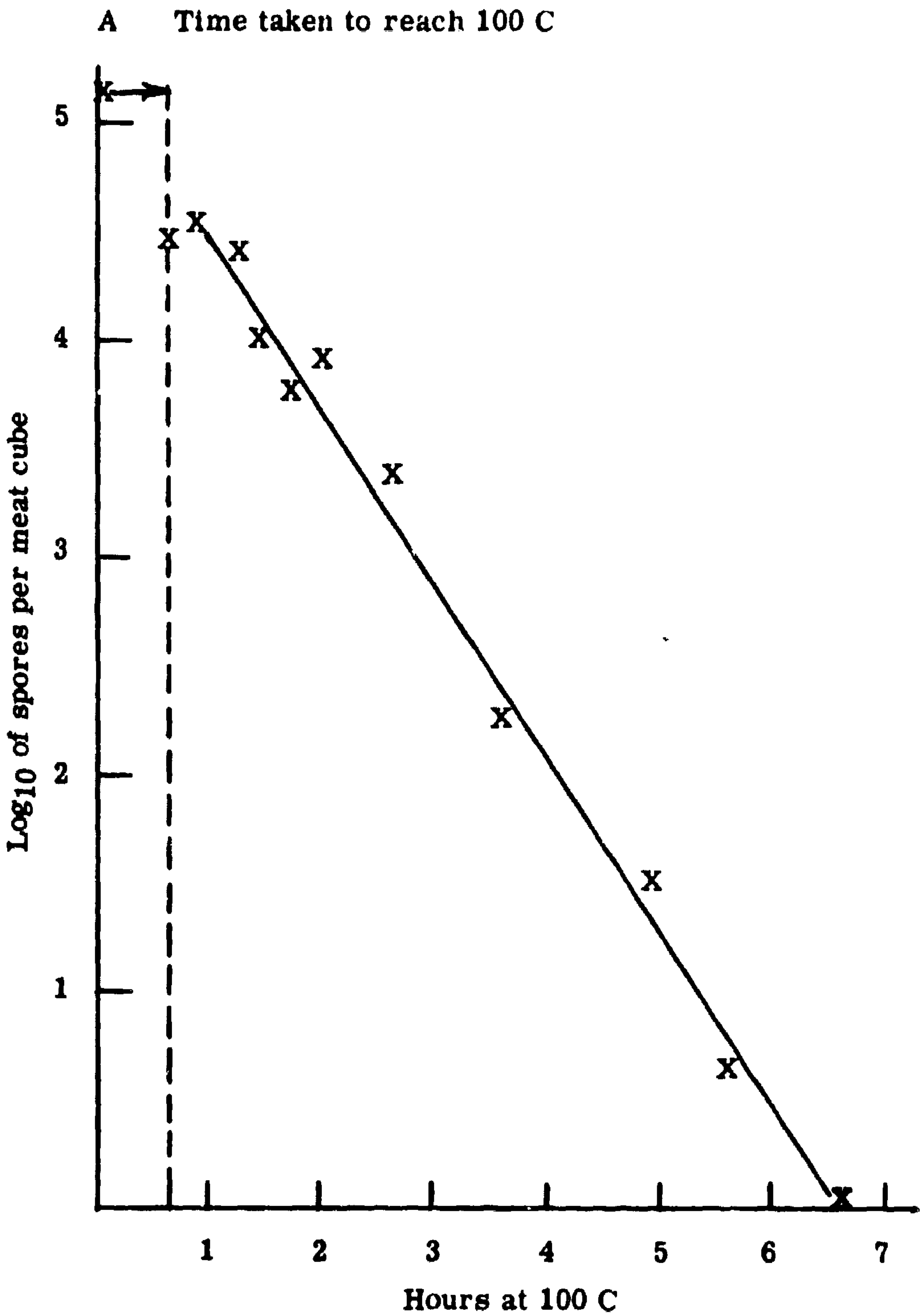

TABLE 1

THE HEAT RESISTENCE OF SPORES OF FOOD POISONING STRAINS OF *Clostridium perfringens*

Culture Code	Approximate number of spores tested	Survival after heating at 100 C for time in minutes 0	15	30	60	90	120	180	240	300
F-4465-61	25,000	+	+	-	-	-	-	-	-	-
F-3278-61	25,000	+	+	+	+	v	-	-	-	-
F-4947-61	1,250	+	+	+	+	v	-	-	-	-
F-1546-52	1,250	+	+	+	+	+	-	-	-	-
F-4022-61	25,000	+	+	+	+	+	+	-	-	-
F-2985-50	500,000	+	+	+	+	+	+	-	-	-
F-2985-50	1,000,000*	+	+	+	+	+	+	+	+	-

v Variable result

* Spores were produced in Robertson cooked meat instead of Ellner medium

TABLE 2

EFFECT OF FREEZING AND STORAGE AT -5 C AND -20 C ON VEGETATIVE CELLS AND SPORES OF *Clostridium perfringens* IN RAW MEAT CUBES

	Storage temperature			
	-5 C		-20 C	
Storage time	Total org per cube	Spores per cube	Total org per cube	Spores per cube
Before blast freezing	120,000	7,900	120,000	7,900
Immediately after blast freezing	13,000	5,500	13,000	5,500
3 weeks	2,500	2,700	11,000	1,500
5 weeks	500	1,600	12,000	1,600
8 weeks	670	670	8,800	1,600
12 weeks		1,600	8,100	2,900
26 weeks	210	1,600	6,600	2,900

NOTE: After obtaining the total count per meat cube the vegetative cells were destroyed by heating the macerate at 85 C for 5 minutes. The heated macerate was counted for spores.

TABLE 3 PART A

EFFECT OF STORAGE TEMPERATURE ON THE VEGETATIVE CELLS AND SPORES OF *Clostridium perfringens* IN RAW MEAT AT 1 C, 5 C, and 10 C

	Storage temperature					
	1 C		5 C		10 C	
Storage time	Total org per cube	Spores per cube	Total org per cube	Spores per cube	Total org per cube	Spores per cube
zero	120,000	7,900	120,000	7,900	120,000	7,900
6 days	51,000	530	53,000	2,700	71,000	1,400
13 days	23,000	570	41,000	6,000	42,000	2,500
28 days	9,300	6,100				

TABLE 3 PART B

EFFECT OF STORAGE TEMPERATURE ON THE VEGETATIVE CELLS AND SPORES OF *Clostridium perfringens* IN RAW MEAT AT 15 C, 20 C, and 37 C

	Storage temperature					
	15 C		20 C		37 C	
Storage time	Total org per cube	Spores per cube	Total org per cube	Spores per cube	Total org per cube	Spores per cube
zero	120,000	7,900	120,000	21,000	120,000	21,000
7 hours					580,000	24,000
15 hours					510,000,000	15,000
2 days	92,000	2,500				
4 days			600,000	15,000		
6 days	64,000	1,500				
12 days			17,000,000	16,000		
13 days	36,000	1,500				

See NOTE Table 2

TABLE 4

EFFECT OF INCREASING TEMPERATURE ON GROWTH RATE OF *Clostridium perfringens* AT pH 5.8 and pH 7.2

Incubation temperature	Time in hours to visible turbidity in RCM broth: pH 5.8	pH 7.2
15 C	672	672
20 C	74	48
25 C	30	24
30 C	24	8
37 C	5	5

TABLE 5

EFFECT OF HEATING AT 70 C FOR 30 MIN ON MEAT CUBES INOCULATED WITH VEGETATIVE CELLS AND SPORES OF *Clostridium perfringens* AND INCUBATED AT 37 C

	RAW MEAT		COOKED MEAT	
Time at 37 C	Total org per cube	Spores per cube	Total org per cube	Spores per cube
zero	64,000	1,500		1,500
1 day	470,000,000	2,800	10,000,000	870
2 days			3,700,000,000	24

TABLE 6 PART A

EFFECT OF INCUBATION AT 37 C ON MEAT CUBES INOCULATED WITH SPORES OF *Clostridium perfringens* PREPARED BY HEATING A BROTH CULTURE AT 85 C FOR 5 MINUTES TO KILL VEGETATIVE CELLS

	RAW MEAT		COOKED MEAT	
Time at 37 C	Total org per cube	Spores per cube	Total org per cube	Spores per cube
zero hours	55,000		24,000	
18 hours	1,000,000,000	26		
20 hours			1,000,000,000	28
66 hours	1,600,000,000	24	5,900,000,000	26

TABLE 6 PART B

EFFECT OF INCUBATION AT 37 C ON MEAT CUBES INOCULATED WITH SPORES OF *Clostridium perfringens* PREPARED BY LYSOZYME TREATMENT TO DESTROY VEGETATIVE CELLS WITHOUT HEATING

	RAW MEAT		COOKED MEAT	
Time at 37 C	Total org per cube	Spores per cube	Total org per cube	Spores per cube
zero hours	620	23,000		32,000
24 hours	1,000,000	18,000	1,000,000	19,000
72 hours	4,200,000,000	17,000	4,800,000,000	10,000

NOTE: Cooking consisted of holding meat at 70 C for 30 min. Meat was cooled before inoculation. By heating at 85 C the spores were heat activated.

TABLE 7

GROWTH OF HEAT TREATED SPORES OF *Clostridium perfringens* IN RAW MEAT STORED AT 20C and 25C

	Storage temperature			
	20 C		25 C	
Storage time	Total org per cube	Spores per cube	Total org per cube	Spores per cube
zero		55,000		55,000
2 days	19,000	160		
3 days	30,000	297	130,000,000	27
7 days	61,000,000	25		
12 days	130,000,000	26		

TABLE 8 PART A

EFFECT OF STORAGE TIME AND TEMPERATURE ON THE LOSS OF HEAT RESISTANCE OF SPORES OF *Clostridium perfringens* AFTER HEATING AT 70 C FOR 30 MIN IN MEAT

	Total org per cube	Spores per cube
Meat cube before heating at 70 C for 30 min	7,700,000	3,300
Meat cubes after heating at 70 C for 30 min		
Cooling time 15 min 32 C*	1,650	165
37 min 18 C*	3,050	170
2 hr 50 min 10.5 C*	6,200	19
Held at 20 C 5 hr	6,200	19
23 hr	110,000	35
Held at 15 C 4 hr	6,700	28
25 hr	5,700	92
Held at 1 C 3-1/4 hr	6,600	21
23 hr	3,800	38
144 hr	4,300	33

Macerate heated at 85 C for 5 min to destroy vegetative cells before counting.
* Temperature at time of counting.

TABLE 8 PART B

EFFECT OF STORAGE TIME AND TEMPERATURE ON THE LOSS OF HEAT RESISTANCE OF SPORES OF *Clostridium perfringens* AFTER HEATING AT 70 C FOR 30 MIN IN RCM BROTH

	Total org per ml	Spores per ml
RCM broth heated at 70 C for 30 min		
Cooling time 40 min 15 C*	94,000	34,000
Held at 15 C 3 hr		27,000
24 hr		28,000
Held at 1 C 24 hr		22,000

Broth heated at 85 C for 5 min to destroy vegetative cells before counting.
* Temperature at time of counting.

PART III

REPORT OF PROCEDURES USED FOR THE ISOLATION OF MICROORGANISMS ASSOCIATED WITH FOOD POISONING

at the

Food Hygiene Laboratory

of the

Public Health Laboratory Service

Colindale, London, England

prepared in connection with a

Secretary of the Army's

Research and Study Fellowship

by

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REPORT OF PROCEDURES USED IN THE FOOD HYGIENE LABORATORY

Summary: Acquisition of the knowledge necessary to examine military food supplies for assurance of the absence of food poisoning microorganisms is important because of the far-reaching protection that can be effected by attaining this objective. Inasmuch as detection of food poisoning microorganisms and allied research is the full time activity of the Food Hygiene Laboratory, London, England, it was an ideal place to study the intricacies of this work. This section is devoted to a presentation of the procedures with which I had some experience there. Some of these were given to me in mimeograph form, some I obtained from notes of employees handbooks and a good deal of the material is based on verbal instructions I received from the technicians who guided me. I have rewritten these in as brief, but useful a manner as practical. In putting these procedures into practice it will be well to augment the information contained herein by consulting the references pertinent to the methods under consideration. These are included in the list at the end of this report.

<table>
<tr><th colspan="2">OUTLINE OF DETECTION OF FOOD POISONING ORGANISMS PART A</th></tr>
<tr><td colspan="2">Comminute 3 to 4 oz of food in a sterile blender or mortar and pestle</td></tr>
<tr><td rowspan="3">Clostridium perfringens</td><td>I. Plate heated cooked food directly on blood agar aerobically and anaerobically. Also plate supernate from Robertson cooked meat aerobically and anaerobically before heating. Incubate 1 to 2 g heated cooked food in Robertson cooked meat 18 hours 37 C. Heat 3 ml supernate 5 min 80 C destroying vegetative cells.</td></tr>
<tr><td>A. Subculture on 2 blood agar plates.
1. Incubate one aerobically 18 hrs 37 C --- serves as aerobic control to verify absence of contaminating aerobes.
2. Incubate one anaerobically 18 hrs 37 C. Observe colonies and type of hemolysis.
B. Pick colony from A 2. Streak across Nagler plate. Incubate 18 hrs 37 C. Half side of Nagler plate contains type A antitoxin of C perfringens. Positive reaction will show inhibition in the opalescence zone on the antitoxin side. Investigate colonial characteristics.
C. Determine serological type.</td></tr>
<tr><td>II. If sample is raw meat, comminute in blender and transfer 20 g to 8 oz jar. Add 100 ml nutrient broth. Steam 1 hr then incubate 24 hrs 37 C. Then proceed as in A, B, and C above.</td></tr>
</table>

OUTLINE OF DETECTION OF FOOD POISONING ORGANISMS PART B	
Comminute 3 to 4 oz of food in a sterile blender or mortar and pestle	
Coliforms and colony counts	I. 10 g in 100 ml 1/4 strength Ringer solution
	A. For colony counts make decimal dilutions of I 1:1,000,000 and carry out Miles-Misra procedure. B. For coliforms place duplicate 1 ml amounts of 1:10 and 1:100 dilutions from A in single strength MacConkey broth and incubate at 37 C 24 to 48 hours. For E coli subculture acid and gas formers according to Mackenzie and Taylor into: 1. brilliant green bile broth 2. peptone water C. Incubate B 1 and 2 at 44 C 18 hrs (for E coli) D. Read results according to Mackenzie and Taylor

OUTLINE OF DETECTION OF FOOD POISONING ORGANISMS PART C	
Salmonella	I. 20 to 25 g in 100 ml selenite-F broth
	II. 20 to 25 g in 100 ml tetrathionate broth
	A. After 24 hours at 37 C 1. Plate I & II on Wilson-Blair agar 2. Plate I & II on Leifson Desoxycholate Citrate agar B. After 72 hours at 37 C 1. Plate I & II again on Wilson-Blair agar 2. Plate I & II again on Leifson Desoxycholate Citrate agar C. Identify select colonies in Gillies tubes
Staphylococci	I. 1 g in Robertson cooked meat containing 10% salt. Incubate 24 hrs 37 C
	II. Plate on nutrient agar containing phenolphthalein phosphate
	A. Ammonia fumes color staph colonies in II pink. B. Coagulase positive Staphylococci colonies are orange pink. Colonies of other strains are pale pink.

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PROCEDURES FOR THE ISOLATION OF FOOD POISONING STRAINS OF *Clostridium perfringens*

SPORES

General: *Clostridium perfringens* will not produce spores *in vitro* in the presence of free fermentable carbohydrate. Low pH may be the causative factor from the rising acidity of fermentation. Classical type A strains occur in the intestines of animals and almost all individuals. *C perfringens* does not sporulate in most foods or in ordinary laboratory media. Spores occur on raw meat from contamination on the surfaces, for, the center of a raw piece of meat is free from spores. A rolled piece of meat has had surfaces exposed before rolling and therefore may be contaminated internally. Spores of *C perfringens*, but not the vegetative cells, survive in water that is chlorinated. This no doubt is a contributing factor to its wide distribution. It is the classical Type A, not the food poisoning strains, that occur so frequently. Spores of food poisoning strains of Type A are more heat resistant than spores of classical Type A strains. Spores of various food poisoning strains can survive 100 C from one to five or more hours. In isolating food poisoning strains of *C perfringens*, non-food-poisoning strains are eliminated in the preliminary heating step, inasmuch as sporulated beta hemolytic strains cannot survive 100 C more than 5 minutes. Smith and Ellner noted a sporulation cycle, while Cash and Collee observed a qualitative increase in heat resistance coincident with spore maturation over the course of 12 hours.

In seeking *C perfringens* in food, an unheated sample and a sample heated at 85 C for 5 minutes should be examined. If only vegetative cells are present they will be found in the unheated sample. The heated sample will reveal spores that require a heat shock for germination. Non-food-poisoning strains produce spores equally as well as food poisoning strains in Ellner medium. However the spores produced in Ellner medium may not be as heat resistant as when they are produced in the intestine or in raw meat. Microscopic observation of spores of *C perfringens* is satisfactory with the Gram staining procedure even when the spores are liberated from the cell body. The spore coat appears as a thinly stained Gram positive halo around the pinkish Gram negative spore body. A more specific stain is the following acid fast spore stain.

Acid fast stain for spores: (after Mackie and McCartney - edited by Cruickshank) Films, which must be thin, are made, dried and fixed with a minimum amount of heating.

1. Stain with Ziehl-Neelsen carbol fuchsin for three to five minutes, heating the preparation until steam rises.

2. Wash in water.

3. Decolorize in a 2 per cent solution of nitric acid in absolute ethyl alcohol. The slide is dipped once rapidly in the solution and immediately washed in water.

4. Counter stain with 1 per cent aqueous methylene blue for three minutes.

5. Wash in water, blot and dry.

The spores are stained bright red and the protoplasm of the bacilli blue.

It should be noted that the spores of some bacteria are decolorized more readily than those of others and that lipid inclusion granules may stain red, appearing like small spherical spores.

BLOOD PLATES

Preparation: Pour a thin layer of 5 to 7 ml of meat digest nutrient agar in a Petri dish and allow it to set. On top of this pour 10 ml of a mixture made at not over 50 C consisting of 5 ml horse blood in 100 ml of the same agar base. Immediately prior to use dry these plates in the incubator at 50 C about 30 minutes. In maintaining aseptic precautions the oven should be cleaned with alcohol starting from the top shelf and proceeding to the bottom. Likewise the plates should be inserted from top to bottom uncovered, right side up, bridging the covers across pairs of plates. Plates are considered dry when water that is condensed on the lid has evaporated. In removing the plates proceed from the bottom shelf to the top. Drying of plates prevents spreading of colonies. An alternate method is to dry about 2 hrs at 37 C, but the longer time increases possibility of contamination.

Hemolysis: As stated in Willis' text (published by Butterworth) theta toxin is produced by all type A strains of *C perfringens* except most of the food poisoning strains. Types B, C, D, and E also produce theta toxin. Hemolysis on horse blood agar is caused by theta toxin. Young colonies of food poisoning strains are therefore nonhemolytic because they do not produce theta toxin. However, after a few days, faint hemolysis may occur. The alpha toxin that is produced by the food poisoning strains is also a hemolysin. It acts on the blood of some animals but not on horse blood cells. Blood agar plates made with horse blood cells therefore serve to differentiate food poisoning from non-food-poisoning strains. Non-food-poisoning strains produce clear zones of beta hemolysis, while food poisoning strains do not.

Examination: In testing for food poisoning strains of C perfringens two blood plates are inoculated from heated cultures. One is incubated as an aerobic control plate. This plate should show no growth since aerobes are expected to be killed during the heating step, although aerobic spores are likely to survive. The other is incubated anaerobically. If a food poisoning strain is present the colonies will be nonhemolytic or slightly alpha hemolytic but not beta hemolytic. Incubation of the blood plates beyond 24 hours may produce some hemolysis where none existed before that time. This could cause difficulty in interpretation. Therefore blood plates should be examined after overnight incubation. Sometimes a rough, vinelike colony occurs which is still C perfringens.

NAGLER PLATES

Preparation: Follow the procedure given in Willis' text on Anaerobic Bacteriology published by Butterworth.

Nagler reaction: The predominant toxin of C perfringens is designated alpha. Alpha toxin is a lecithinase which acts on the lipoprotein complex of the egg yolk or human blood serum component of Nagler medium. In this medium a zone of opalescence is produced by alpha toxin which is liberated by all types and strains of C perfringens. This toxin is specifically neutralized by antiserum of C perfringens. Thus, a positive Nagler reaction is inhibited by alpha antitoxin. Use is made of this property in the Nagler reaction to verify presumptive findings of C perfringens on anaerobic blood plates. About 0.1 ml of this antitoxin, spread over half of a Nagler plate, serves as a control. Fairly dry plates are used so that the antitoxin does not overspread. To assure this, after applying the antitoxin, the plates are dried before inoculation for about 10 minutes at 37 C. A streak of inoculum across the untreated and treated half of a Nagler plate incubated overnight reveals the presence or absence of C perfringens. Colonies on the untreated part show a marked zone of opalescence; whereas colonies on the treated half have no zone of opalescence since action of the lecithinase on phospholipid has been inhibited by lecithinase antitoxin. Other organisms producing this reaction, although less intensely, are C bifermentans and C sordellii. These, however, are easily distinguished from C perfringens by their colonial appearance, which is an important differentiating factor, by their inability to ferment lactose, by their strong odor from proteolytic action, and by their ready sporulation. Consequently Nagler medium containing lactose and a fermentation indicator such as recommended by Willis and Hobbs is diagnostic of C perfringens. Furthermore the two interfering Clostridia would not respond to serological typing with C perfringens antisera Nr 1 to 13. It should also be borne in mind that classical type A beta-hemolytic strains of C perfringens

will not agglutinate with antisera 1 to 13 prepared against food poisoning strains.

Type A toxins: Besides alpha toxin, some food poisoning strains produce deoxyribonuclease; some collagenase; some hyaluronidase. Other major toxins are not present in food poisoning strains.

IDENTIFICATION OF *Clostridium perfringens* PRESENT IN THE SPORULATED STATE IN RAW MEAT

1. Three to four ounces of a sample suspected of containing spores, such as raw meat, is heated in nutrient broth 1 hr at 100 C.

2. From the nutrient broth inoculate on two blood agar plates. Incubate 18 hrs at 37 C one anaerobically, the other aerobically. From Robertson cooked meat medium heat 3 ml of supernate 5 minutes at 80 C to kill vegetative cells. Inoculate on two blood agar plates. Incubate 18 hrs at 37 C, one anaerobically, the other aerobically.

3. If anaerobic culture is not pure, replate on blood agar in presence of 1 per cent neomycin to eliminate contaminants. Otherwise omit this step. All types of *C perfringens* will grow on blood or Nagler plates containing 50 ug/ml neomycin for inhibition of contaminants. Nonhemolytic, heat resistant, type A strains tolerate 100 ug/ml.

4. From a typical colony streak on Nagler plate, half of which is covered with *C perfringens* type A alpha antitoxin. Incubate in a McIntoch-Fildes jar 18 hrs at 37 C.

5. Determine the serological type. Positive agglutination reactions are obtained with thirteen antisera of specific serological types prepared from the common food poisoning strains.

IDENTIFICATION OF *Clostridium perfringens* PRESENT IN THE VEGETATIVE STATE IN COOKED FOODS

1. Observe for characteristic morphology of Gram stained cells.

2. Comminute aseptically and plate on blood agar. Incubate aerobic control and anaerobic plates. Look for typical colonies and determine type of hemolysis. *C perfringens* colonies are 1 to 5 mm in diameter; not white, but colorless or translucent; conical shape; may have rough edges.

Colonies are odorless which differentiates them from C bifermentans, which emit a strong proteolytic odor. C bifermentans, unlike C perfringens does not ferment lactose.

3. Inoculate 3 to 4 grams into Robertson cooked meat medium and incubate 24 hrs at 37 C.

4. Subculture from anaerobic colony from 2, or if negative from 3 on Willis-Hobbs lactose, egg yolk, milk, agar plate, one half of which is controlled with type A antitoxin for Nagler reaction.

5. Subculture from 2 or 3 into litmus milk tube for stormy fermentation test for lactose and observe again for characteristic morphology of Gram stained cells. As the reaction with litmus milk is variable the stormy fermentation is not always produced.

6. To prove that these vegetative cells are food poisoning strains subculture from 2 or 3 into Robertson cooked meat medium to obtain active growth.

7. Inoculate from 6 into Ellner medium to produce spores.

8. Observe with acid fast spore stain and verify ability to withstand 100 C one hour in Robertson cooked meat medium.

9. Verify toxicologically by growing the cells in nutrient broth. Prepare two cultures. Test one after 5 hours, the other after 24 hours incubation by applying the Oakley-Warrack typing technique of 1953 to the filtrate.

PREPARATION OF *Clostridium perfringens* SEROTYPES

Purpose: Agglutination reactions are carried out with 13 antisera prepared from the common food poisoning strains. The 13 antisera types are significant for epidemiological studies in food poisoning outbreaks. Antisera of C perfringens are not available commercially. The antisera made in horses have lower titers and more cross reactions than antisera made in rabbits. In the Food Hygiene Laboratory in London these antisera are produced in rabbits in quantities sufficient only for the Laboratory's own needs. The procedure used in Colindale is given below.

Preparation of Inoculum: To expel air boil 200 ml of nutrient broth containing one per cent glucose. Inoculate with an overnight culture of C perfringens grown at 37 C. Separate the cells by centrifugation washing twice with distilled water. Resuspend in physiological saline solution to give

a turbidity of 20,000,000,000 organisms per ml. Add 0.1 ml of 40 per cent formalin to 25 ml of the suspension and let the culture stay at room temperature for 2 days. Test for sterility. Store at 4 C until required for inoculation.

Initial tests for antibodies: Draw 5 ml of blood from the rabbit's ear. Allow the blood to stand overnight at room temperature. Separate the serum by centrifugation. Make double dilutions of this serum. Test the serum for null agglutination with standard stock suspensions of serotypes 1 to 13. Also test this serum for null agglutination of strains to be inoculated for production of antisera. Test new strains with all 13 antisera to detect any cross agglutination.

Scheme for inoculation:

1st day withdraw 5 ml blood from rabbit for null tests
3rd day inject 0.1 ml inoculum intravenously
5th day inject 0.2 ml inoculum intravenously
7th day inject 0.4 ml inoculum intravenously
9th day inject 0.8 ml inoculum intravenously
11th day inject 1.0 ml inoculum intravenously
13th day inject 1.0 ml inoculum intravenously

On the 15th day withdraw 5 ml blood from the rabbit and determine the titer. If low, boost the titer with further doses of 1.0 ml inoculum. When the potency is adequate harvest the blood, separate its serum by centrifugation, and preserve.

PREPARATION OF BACTERIA FREE FILTRATE OF *Clostridium perfringens* FOR TOXIN INVESTIGATION

Procedure: Boil 100 ml of Hartley (p 192 of Mackie & McCartney by Cruickshank) digest medium for a few minutes to expel oxygen. Cool to 45 C. Inoculate with 0.5 ml of an overnight culture of C perfringens which has been tested for purity. Incubate 6 hrs 37 C. Plate to again test for purity. Add 0.5 g diatomaceous silica filter aid to the broth, mix, and let stand for a few minutes. Spin down to pack the cells. Filter the supernate through large filter paper, then through a Seitz filter.

Test the filtrate for sterility. If sterile, it is ready for toxin study. The six types of C perfringens are toxicological types and are determined by the specific toxins that each type produces. In all, 12 distinct toxins are produced, several by each type. These toxins and their characteristics are tabulated completely on pages 144 and 145 of Willis' text on anaerobic bacteriology published by Butterworth in 1960.

PROCEDURES FOR THE ISOLATION AND IDENTIFICATION OF *Escherichia coli* TYPE I

Brilliant green bile broth:

Bacto Oxgall	30 g
Bacto Peptone	15 g
Lactose	15 g
Bacto Brilliant Green (1 per cent solution)	2 ml
Water to 1500 ml	
pH 7.2 - 7.4	

Transfer 5 ml into test tubes containing Durham tubes.

Presumptive test with MacConkey broth: Prepare MacConkey broth fermentation tubes containing Durham tubes and bromcresol purple indicator. To two pairs of these tubes transfer 1 ml in duplicate of 1:10 and 1:100 dilutions of sample. Incubate at 37 C for 48 hrs, reading at 24 hrs and 48 hrs. Production of gas and acid, shown by the indicator change to yellow is presumptive of the presence of coliforms.

Verification with brilliant green bile broth: From each MacConkey tube showing acid and gas subculture into tubes containing peptone water and into fermentation tubes containing brilliant green bile broth with Durham tubes. Incubate 24 hrs 44 C. If tubes in brilliant green bile broth show gas production then test for indole in the corresponding tube containing peptone water. Add 1 ml ether to the peptone water tube, shake to dissolve the indole and add a few drops of Ehrlich rosindale reagent. This reagent should be layered under the ether. A pink ring at the junction of the layers is a positive test for indole and indicates presence of Type I *E coli*. Only Type I *E coli* gives positive indole, and gas in brilliant green bile broth after incubation at 44 C.

Interpretation according to Mackenzie-Taylor:

Gas in brilliant green bile broth	Indole production in peptone water	Organisms indicated
+	+	*E coli* Type I
+	o	Irregular Type II or VI
o	+	Other coliforms
o	o	Other coliforms

PROCEDURES FOR THE ISOLATION AND IDENTIFICATION OF *Salmonella*
TETRATHIONATE ENRICHMENT BROTH FOR ISOLATION OF *Salmonella*
(after Rolfe)

Formula:

Difco proteose peptone	5 g
Bacto bile salts	1 g
Calcium carbonate	10 g
Sodium thiosulfate	30 g

Suspend 4.6 g of this dehydrated medium in 100 ml of water. Heat to boiling. Cool to 45 C. Add 2 ml of the following iodine solution:

Iodine crystals	6 g
Potassium iodide	5 g
Water	20 ml

Add 1 ml brilliant green 1:1000.

In practice at the Food Hygiene Laboratory tetrathionate enrichment broth is prepared as follows:

Solution A:

Iodine crystals	400 g
Potassium iodide	500 g
Water	2 liters

Solution B:

$Na_2S_2O_3 \cdot 5H_2O$	25 g
Water	130 ml

Used as follows:

110 ml A + 220 ml B + 2 ml of 1 per cent Difco brilliant green.

This yields 332 ml of concentrated tetrathionate broth which is added to 1 liter of nutrient broth consisting of:

Lab Lemco Beef Extract	1 per cent
Evans peptone	1 per cent
$CaCO_3$	In excess

The volume of the final medium is 1332 ml. This final solution is 2X, or double strength. It is to be used diluted 1 in 2 with Ringer solution.

GPO 809-110-11

Salmonella on MacConkey agar: All Salmonella species except S ferlac do not ferment lactose. Organisms that ferment lactose turn neutral red a deep red which is negative for Salmonella. However S ferlac, a species apparently found only in coconut, turns neutral red a faint red meaning that it is not a vigorous lactose fermentor. A yellow or faint yellow coloring of MacConkey agar is due to non-lactose fermentors. Colonies on such plates are suspect of being Salmonella and require further testing with Gillies tubes.

Wilson and Blair bismuth sulfite agar for Salmonella: NOTE: Do not confuse this medium with the Wilson and Blair medium specifically designed for isolation of C perfringens. The latter medium is not used in the Food Hygiene Laboratory or other laboratories visited in Great Britain because procedures using Robertson cooked meat medium, blood agar, and Nagler media are more reliable for isolation and identification of this anaerobe. For isolation of Salmonella use Difco bismuth sulfite agar according to directions given in the Difco manual excepting the statement, "This medium should be prepared the day it is used." Fresh medium inhibits the majority of Salmonellae. The medium should be prepared at least 24 hours before it is used and should be stored at 4 C not more than 2 weeks.

Desoxycholate citrate agar for Salmonella: Use Leifson medium as modified by Hynes given on pages 218 to 220 in Mackie and McCartney, edited by Robert Cruickshank.

IDENTIFICATION OF Salmonella

Gillies Nr 1 tube: This is a green medium used as a butt and slant in the same tube. The medium contains urea, glucose, and mannitol. The latter 2 sugars are fermented by Salmonella. These organisms turn the green medium yellow due to acid production from the sugar fermentation but do not react with urea. If urea is hydrolyzed the medium turns blue indicating an organism that is not a Salmonella. Mannitol reacts in the slope, glucose in the butt.

Gillies Nr 2 tube: This is a butt containing sucrose and salicin, which are not fermented by Salmonella. Touch a Salmonella suspect colony with a wire tip and insert the wire into the center of the medium to a depth of one half inch only. If the organism is a Salmonella the top half inch remains a bluish green unchanged. The medium below the half inch penetration becomes cloudy due to motility and multiplication of the Salmonella. Two test papers are secured suspended apart from each other inside the top of the tube with a cotton plug. One of these is impregnated with lead acetate for H_2S detection. The other is an indole indicating paper containing Ehrlich reagent, which turns pink if indole is present. Salmonellae do not produce indole but most strains produce H_2S.

Technique applied in inoculation of Gillies tubes: Touching a suspect colony with a straight wire inoculate a trace on a MacConkey plate. Continuing with the same transfer inoculate a trace in the test tube containing nutrient broth by tilting the tube and touching the wire on the exposed wall of the tube which is below the broth surface when the tube is vertical. Then insert the wire 1/2 inch into Gillies Nr 2 tube. Next, streak it upward in a straight line along the center of the slope of Gillies Nr 1 tube; insert it all the way down the butt of this tube and spread the streak on the slope while withdrawing the wire. Finally with a loop spread the inoculum placed initially on the MacConkey plate. Thus by touching a select colony with the wire and using it to inoculate four media, an identical transfer is assured. The Gillies tubes and broth are inoculated before the transfer is spread on the MacConkey plate to avoid the possibility of picking up a contaminant from the plate. After the inoculation is completed insert the two strips of indicator paper into Gillies Nr 2 tube. Incubate these four media 18 to 24 hours at 37 C.

GILLIES MODIFICATION OF KOHN MEDIUM FOR SALMONELLA AND SHIGELLA

(This medium is available from Oxo Ltd)

Medium I

Evans peptone	15 g
Yeastrel	4 g
glucose	1 g
mannitol	10 g
agar	16 g
Singer indicator mixture	26.5 ml
distilled water	1000 ml
pH = 7.2	

Medium II

agar	4 g
Evans peptone	10 g
Difco tryptone	10 g
NaCl	5 g
$Na_2HPO_4 \cdot 12H_2O$	0.25 g
sucrose	10 g
salicin	10 g
bromthymol blue	0.01 g
$Na_2S_2O_3 \cdot 5H_2O$	0.025 g
distilled water	1000 ml
pH = 7.4	

Singer indicator mixture for differentiation of urease producing and alkali forming organisms consists of 3 separate 0.2 per cent indicator solutions prepared as follows:

Indicator		N/20 NaOH	Water
1. bromthymol blue	0.20 g	6.4 ml	100 ml
2. cresol red	0.20 g	10.6 ml	100 ml
3. Thymol blue	0.20 g	8.6 g	100 ml

Prepare 26.5 ml of Singer indicator by mixing the separate solutions in the following proportions:

bromthymol blue	12.5 ml
cresol red	4.0 ml
thymol blue	10.0 ml

The prepared indicator keeps well.

Preparation of Medium I

1. Evans peptone) dissolve in given volume of distilled water.
   Yeastrel)
2. adjust reaction to pH = 7.8 (phenol red method)
3. boil for 20 minutes. Filter
4. adjust reaction to pH 7.2
5. measure volume, then add agar 1.6 per cent
6. autoclave for 10 minutes at 10 lbs
7. add mixture of glucose 0.1 per cent and mannite 1.0 per cent
8. add indicator mixture (26.5 ml)
9. distribute to bottles, and sterilize for 15 minutes at 10 lbs and store in this form

For use

10. melt, cool to 60 C
11. add 50 ml of sterile 20 per cent urea solution per litre
12. distribute aseptically in sterile test tubes to a depth of 6.5 cms, allow to solidify to give a butt of 2.5 cms.

Preparation of Medium II

1. Add all ingredients except sugars and indicator
2. adjust reaction to pH 7.8
3. boil for 10 minutes, then filter
4. adjust reaction to pH = 7.4 (phenol red method)
5. measure volume
6. add sucrose, salicin and indicator
7. autoclave for 15 minutes at 15 lbs pressure
8. distribute aseptically into sterile test tubes in 8 ml amounts, allow to set in vertical position

Test Papers

1. Strips of filter paper are impregnated with saturated lead acetate solution; dry in oven at 70 C

2. Indole test papers are impregnated with a solution of 5 g p-dimethyl-aminobenzaldehyde; 50 ml methanol; 10 ml phosphoric acid. Dry at 70 C for minimum period. This solution must be dropped on to the filter paper - DO NOT IMMERSE THE PAPER IN THE SOLUTION.

METHOD OF TESTING

Material from a single non-lactose-fermentor from the primary isolation plate is inoculated with a long straight wire into

1. Medium I, a) smear the slant, b) stab base of butt.
2. Medium II, single stab into upper 1/2 inch, do not exceed.
3. Wash needle in tube of peptone water, incubate during day, plate in the evening to check for purity.
4. Suspend H_2S and indole test papers over medium II held separated in place by a cotton plug. These two reagent papers must not touch each other.
5. Incubate overnight at 37 C.

READING OF TEST

Medium I	yellow butt: glucose fermentation yellow slant: mannitol fermentation deep blue throughout: urea positive split along wire or disruption of the medium: gas production
Medium II	blue around top 1/2 inch: no fermentation of sucrose and/or salicin yellow around top 1/2 inch or throughout medium: sucrose and/or salicin fermentation disruption of medium: gas production lead acetate paper black: H_2S positive indole paper pink: indole positive, obscured if heavy fermentation in tube II fuzzing from stab: motile strain

Additional tests for *Salmonella*:

1. Inoculate a dulcite sugar tube. Most *Salmonella* produce acid and gas in dulcite.

2. Incubate in peptone water and inoculate two drops in malonate. Fermentation is shown by a color change from green to blue indicating absence of *Salmonella*.

3. Inoculate one drop of nutrient broth culture with KCN solution. *Salmonella* do not react with KCN.

Each organism provisionally identified as:

1. Salmonella
2. Shigella (differentiate between mannite and non-mannite fermentors)
3. "non-pathogenic"

CONFIRMATION:

Confirm by usual serological techniques which can be carried out on material from these media.

Identification of *Salmonella* serotypes: By antigenic analysis according to the Kauffman-White scheme the *Salmonella* are subdivided into about 600 serotypes. In this scheme the somatic and flagellar antigens are specified for each serotype. When these antigens are determined by slide agglutination tests on an unknown species that responds to the biochemical reactions of the genus *Salmonella*, the organism is fully identified. The process consists of determining the "O" and the "H" antigens. Identification of the somatic or "O" antigen places any member of the genus in one of a number of groups each designated by the letters A through I. Determination of the serological character of the flagella or "H" antigen provides the additional information required to type the organism completely. The latter requires identification of the phasic constitution of the flagella. *Salmonella* "H" antigens occur in two different forms or phases--specific and non-specific. Some species are monophasic, occurring either in the specific or non-specific form. Others may occur in both forms. The individual bacterial cells do not contain both forms of H antigens. A portion of the cells contain one form and the remainder the other.

Analysis of phase variation in a species is accomplished with the aid of a Craigie tube. This consists of a test tube containing about 5 ml of very soft nutrient agar. A small glass inner tube open at both ends is inserted in this agar. The tube is of such length that the upper end projects above the surface of the agar. While the agar is liquid but cooled to 45 C, a few drops of sterile nonspecific phase serum is introduced into the small tube. The set up is allowed to solidify upright after which the small tube is inoculated with the nonspecific culture. After incubation, the specific form is recovered by subculture from the surface of the agar surrounding the small tube. Since a high proportion of the organisms inoculated in the small tube are in the nonspecific phase they will be agglutinated by the serum introduced previously.

However, those in the specific phase will remain unscathed, will multiply, and through their motility will migrate beneath the bottom of the small tube and outside it upwards to the surface of the surrounding agar.

An alternative procedure to the Craigie method features a MacConkey plate with a 1/4 inch agarless channel across it made by cutting out the agar. Strips of filter paper impregnated with the requisite serum are bridged across the channel. The nonspecific inoculum is applied on one side and the plate incubated. Unagglutinated *Salmonella* move across the bridge and multiply on the opposite side of the channel in changed phase.

FORMULAE FOR SINGLE STRENGTH SELENITE MEDIUM FOR ISOLATION OF *Salmonella*

SELENITE-F

Sodium hydrogen selenite ($NaHSeO_3$)	0.4 g
Mannitol	0.4 g
Peptone	0.5 g
Sodium hydrogen phosphate (Na_2HPO_4)	1.0 g
Distilled water	100 ml

pH adjusted to 7.0
Sterilized by filtration or steaming for 1/2 hour

CONCENTRATED 8X SELENITE ENRICHMENT MEDIUM FOR ISOLATION OF *Salmonella*

Sodium acid selenite	80 grams
Peptone	100 grams
Mannitol	80 grams
Na_2HPO_4	190 grams
NaH_2PO_4	10 grams
Water	2500 ml

In the preparation of this medium the water must not be heated beyond 65 C. Sterilize by passing through a Seitz filter. Dilution of the 8-fold concentrated solution is accomplished as follows: A series of bottles is prepared containing 750 ml sterile water in each. Into each is added 250 ml of the 8X solution. A syphon arrangement is convenient for this transfer. At this stage the concentration is 2 X. This is adjusted to 1 X with Ringer solution at the time of use, allowing for the water contained in the sample.

Examples:

Liquid eggs or other fluid samples	25 ml
Ringer solution	25 ml
2 X selenite	50 ml
Dried eggs; coconut; meat; shrimp	25 g
Ringer solution	50 ml
2 X selenite	50 ml

PROCEDURES FOR THE ISOLATION AND IDENTIFICATION OF STAPHYLOCOCCI

PHENOLPHTHALEIN PHOSPHATE AGAR PLATES

Nutrient agar:

	Per cent
Lab Lemco Beef Extract	1.0
Peptone	1.0
Salt	0.5
Agar	1.5
Adjust to pH 7.4 and sterilize	

Phenolphthalein diphosphate stock solution:

Phenolphthalein diphosphate	0.5 g
Distilled water	100 ml
Sterilized by filtration	

Preparation on plates

Melt and cool 100 ml nutrient agar

Add 2 ml 0.5 per cent phenolphthalein diphosphate and 0.25 ml aerosporin solution (63.3 mg polymixin B sulfate in 10 ml saline) for inhibition of Gram negative organisms. Pour the mixed components directly into sterile Petri plates and dry the plates for 1 hr at 37 C without further sterilization of the mixture. Prepared plates are considered to be aseptic because they are made from three sterile components. Store plates at 4 C.

Phenolphthalein phosphate test for *Staphylococcus*: *Staphylococcus* organisms may be present in cooked meat, shrimps, cheese, and other foods. Transfer one gram of sample into Robertson cooked meat medium containing 10 per cent salt. Incubate 24 hours 37 C to develop the *Staphylococci*

and suppress other organisms. Streak one loopful on a nutrient agar plate containing phenolphthalein phosphate and aerosporin. Test for the liberation of phenolphthalein by phosphatase of Staphylococci by its color reaction with ammonia. Put a few drops of concentrated NH_4OH into the inverted lid of the Petri dish. Expose the half containing the culture over the ammonia for a few minutes. The fumes react with the phenolphthalein to give a deep pink color on and around the colonies. S aureus and S pyogenes are both phosphatase positive.

Coagulase test for Staphylococcus aureus: The production of coagulase is characteristic of S aureus. Pick a deep pink colony from the phenolphthalein phosphate agar plate with a wire and inoculate a trace into 5 ml of nutrient broth by touching the wall of the slanted tube at a point below the surface of the broth when the tube is upright. With the same wire inoculate a nutrient agar plate spreading the inoculum with a loop. Incubate 24 hours 37 C. Make a 1:10 dilution of human blood plasma or rabbit plasma. Distribute 0.5 ml volumes into small diameter test tubes. From the nutrient broth add a few drops of the Staphylococcus suspension into the plasma tubes. Incubate 2 hours 37 C. Observe if the fibrin in the plasma is coagulated. If it is coagulated S aureus is indicated as it is the only member of this genus that is coagulase positive. This finding is verified by phage typing in a laboratory specializing in this technique as described by Munch-Petersen.

QUANTITATIVE ISOLATION OF COAGULASE POSITIVE Staphylococci FROM CHEESE

Ten grams of cheese are weighed in a sterile jar, transferred to a sterile mortar, and emulsified gradually using a sterile pestle and 50 ml quarter strength Ringer solution ending up with a suspension of the cheese in 100 ml of Ringer. Dilutions are made in the usual way and count is done by the Miles and Misra technique on phenolphalein phosphate agar. Put six drops on each plate, duplicating the dilutions up to 1 in a million.

ISOLATION OF SALMONELLAE AND COLIFORMS FROM COCONUT

Into each of two 8 oz jars transfer a layer of coconut 3/8 to 1/2 inch in depth, or about 20 to 25 grams. Into one add 100 ml of nutrient broth and into the other 100 ml of selenite broth. The latter consists of 50 ml of quarter strength Ringer solution plus 50 ml of double strength selenite solution. Incubate at 37 C. After 24 hours both enrichment cultures are plated on Leifson's desoxycholate citrate agar and Wilson and Blair agar. In order to detect Salmonellae that may be present in small numbers these two cultures are again plated on these two agars after 72 hours. The plates are incubated at 37 C for 48 hours.

GPO 809-110-10

In practice, to conserve plates and media, plate a loopful of the nutrient broth culture to one half side of a Leifson plate, and a loopful of the selenite broth culture to the other half side. Likewise, from each of the broth cultures, plate each half of a Wilson-Blair plate. Half platings are adequate for dried foods such as powdered eggs and desiccated coconut. Whole plates are more desirable for liquid eggs, meat, fish, or shrimps. Suspect colonies are selected from the incubated plates for fermentation reactions in Gillies' modification of Kohn's medium, and also for serological examination.

Colony counts are made by the Miles-Misra technique on blood agar. The presence of Escherichia coli in 1:10 and 1:100 dilutions is determined by incubation in MacConkey broth at 37 C; followed by subculture into brilliant green bile broth and peptone water with incubation at 44 C.

DETECTION OF SALMONELLAE IN EGG PRODUCTS

A. ISOLATION

1. Whole egg mix - frozen or liquid

Two 25 gm amounts of liquid, frozen or thawed sample are weighed into sterile jars with screw caps. After the addition of 25 ml of quarter strength Ringer solution, the mixes are incubated for 1 to 2 hours at 37 C before the addition of 50 ml double strength selenite to one lot and 50 ml double strength tetrathionate to the other. These enrichment cultures are incubated at 37 C and subcultured on desoxycholate citrate agar, and on Wilson and Blair agar after 24 hours, and again after 3 days incubation. Suspect colonies are picked from the plates after 30 to 48 hours incubation and examined as directed above under IDENTIFICATION.

2. Spray dried whole egg powder

Thirty grams of powdered egg are mixed gradually with 150 ml of nutrient broth; and 20 gm of the powder are mixed with 50 ml of quarter strength Ringer solution and 50 ml of double strength selenite. Both enrichment cultures are incubated at 37 C. After 24 hours, and if necessary after 3 days also, both enrichment cultures are plated on Leifson's desoxycholate citrate agar, and Wilson and Blair agar. Suspect colonies are picked from the plates after 30 to 48 hours incubation and examined as directed above under IDENTIFICATION.

3. Flaked and powdered egg albumen

Two 25 gm amounts of albumen are weighed into sterile jars with screw caps. 125 ml quantities of nutrient broth are added to each jar, shaken well and incubated at 37 C. After 1 to 2 hours incubation each jar should be reshaken.

These enrichment cultures are plated on desoxycholate citrate agar, and Wilson and Blair agar after 24 hours and again after 3 days at 37 C. The plates are incubated for 48 hours at 37 C.

B. IDENTIFICATION

Suspect colonies are picked into peptone, lactose, and urea media. After a few hours incubation the peptone water culture may be used to inoculate further media for fermentation tests. Alternatively, suspect colonies are picked into peptone water and two Gillies tubes. Organisms giving the correct fermentation and other biochemical reactions are investigated serologically.

ISOLATION OF *SALMONELLAE* FROM MEAT SAMPLES

Meat is removed from container on to a sterile tray and pieces are cut from the outside of the meat, using sterile forceps and scissors. The pieces are placed into two sterile screw-cap jars. One hundred ml of selenite is added to one jar and 100 ml tetrathionate to the other. The jars are incubated at 37 C and plated on to Wilson-Blair and Leifson agar after one day, and again after 3 days incubation. Suspect colonies are subcultured into Gillies tubes for biochemical reactions and agglutination tests described under "egg products."

BACTERIOLOGICAL EXAMINATION OF FROZEN COOKED PRAWNS AND SHRIMPS

Preparation of samples: The prawns are allowed to thaw for a few hours at room temperature before 50 grams are minced in an Atomix.

Colony and coliform counts: Ten grams of the minced sample is suspended in 100 ml of quarter strength Ringer solution and appropriate dilutions are made usually up to 1:1,000,000. Colony counts are carried out by the surface drop technique on blood agar, and the plates incubated for 2 days at 37 C before counting. Yeastrel milk agar pour plates can also be used.

For coli-aerogenes counts duplicate 1 ml amounts of the 1:10 and 1:100 dilutions are placed in single strength MacConkey broth and incubated at 37 C for 24 to 48 hours. Tubes producing acid and gas are subcultured into brilliant green bile broth and peptone water to test for indole production, and incubated at 44 C for detection of *Escherichia coli* I.

Examination for *Salmonella*: Approximately 25 gram amounts of the minced prawns are put into 100 ml of selenite F medium containing mannitol instead of

lactose, and 100 ml of tetrathionate broth. The liquid enrichment cultures are plated on Wilson-Blair agar and on desoxycholate citrate agar after one day, and again after 3 days incubation at 37 C. Suspect colonies are subcultured into Gillies tubes for fermentation reaction and serological identification, and at the same time on MacConkey agar for purity and lactose fermentation.

Examination and count for coagulase-positive *Staphylococci*: One fiftieth ml of a 1:10 dilution of the food is spread over the surface of blood agar; or 7.5 per cent salt nutrient agar; or phenolphthalein phosphate agar; or other suitable medium. The number of colonies developing will give an approximate count of the number of *Staphylococci* present in the food. For enrichment cultures approximately one gram of the sample should be incubated in Robertson cooked meat containing 10 per cent salt. After overnight incubation the culture is plated on 7.5 per cent salt nutrient agar; or phenolphthalein phosphate agar; or other suitable medium. Likely colonies are examined for coagulase production and if positive they are phage typed.

The surface count on blood agar plates can also be used for the enumeration of *Staphylococci* when the count is too high to estimate from 1:10 dilution only.

BACTERIOLOGICAL EXAMINATION OF WATER

THE PRESUMPTIVE COLIFORM COUNT

Apparatus:

1. Sterilized glass bottles provided with a ground glass stopper having an overlapping rim should be used.

   If the water to be sampled is likely to contain traces of chlorine or chloramine a quantity of sodium thiosulphate to neutralize these substances should be added to the bottles before sterilization.

2. 10 ml graduated pipettes.
3. 1 ml graduated pipettes.
4. 37 C incubator or water bath with thermometer graduated in degrees C.
5. 44 C ± 0.25 C water bath with thermometer graduated in tenths of 1 C.

Reagents:

1. Bottles containing 50 ml	D/S MacConkey lactose bile salt broth	)	with Durham tubes
2. Tubes containing 10 ml	D/S MacConkey lactose bile salt broth	)	
3. Tubes containing 5 ml	S/S MacConkey lactose bile salt broth	)	
4. Tubes containing 5 ml	Brilliant Green Bile broth	)	

5. Tubes containing 5 ml Peptone water

6. Standard nutrient agar in 20 ml volumes

7. 1/4 strength Ringer solution.

Sample: The sample bottle should be inverted 25 times to distribute any deposit uniformly throughout the water.

Inoculation in MacConkey broths:

1. Good quality water (drinking water supply)

   1 x 50 ml D/S MacConkey broth + 50 ml water
   5 x 10 ml D/S MacConkey broth + 10 ml water

2. Medium quality water (treated water in storage tanks)

   1 x 50 ml D/S MacConkey broth + 50 ml water
   5 x 10 ml D/S MacConkey broth + 10 ml water
   5 x 5 ml S/S MacConkey broth + 1 ml water

3. Poor quality water (untreated water)

   5 x 10 ml D/S MacConkey broth + 10 ml water
   5 x 5 ml S/S MacConkey broth + 1 ml water
   5 x 5 ml S/S MacConkey broth + 1 ml of 1/10 dilution of water* in 1/4 strength Ringer solution

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* Tap water may be used if it has been shown to be free from germicidal activity. Glass distilled water may be used, but not water from a metal still.

STANDARD PLATE COUNT

Method: Introduce samples into duplicate sterile Petri plates using 1 ml of undiluted water and 1 ml of serial dilutions. Add 10 to 12 ml melted and cooled nutrient agar. Incubate plates at 37 C for 24 hours. All tubes and bottles are incubated at 37 C for 48 hours, reading at 24 hours and 48 hours. Any tube or bottle showing acid and gas is subcultured into a tube containing 5 ml Brilliant Green Bile Broth and also into a peptone water tube. These subcultures are incubated at 44 C ± 0.25 C for 24 hours.

Observing and Reporting Results: The numbers of tubes and bottles of MacConkey broth incubated at 37 C and showing acid and gas after 48 hours incubation are recorded and the presumptive coliform count reported as the probable number of coliforms per 100 ml of water. Tubes incubated at 44 C for 24 hours showing growth and gas in the Brilliant Green Bile Broth and indole positive from the peptone water are recorded and the count reported as probable number of coliforms per 100 ml of water. Counts on plates having between 30 and 300 colonies should be made, the average taken and reported as number of colonies (not organisms) per ml. Dilutions having more than 300 colonies should be discarded.

Differentiation of the Coliform Group

All of these organisms are capable of producing acid and gas from lactose peptone water in 48 hours at 37 C.

	Gas in lactose bile salt medium at 44 C	Indole	M R	V P	Growth in citrate
Bact coli type I (Escherichia coli)	+	+	+	-	-
Bact coli type II	-	-	+	-	-
Intermediate type I	-	-	+	*	+
Intermediate type II	-	+	+	*	+
Bact aerogenes type I	-	-	-	+	+
Bact aerogenes type II	-	+	-	+	+
Bact cloacae	-	-	-	+	+
Irregular type I	-	+	+	-	-
Irregular type II	+	-	+	-	-
Irregular type VI	+	-	-	+	+
Irregular other types	v	v	v	v	v

* Usually negative, but weak positive reactions are occasionally found.
v Variable reactions

ASSAY OF DAIRY PRODUCTS FOR NISIN

(Example with cream)

Tomato dextrose agar:

Evans peptone	15 g
Glucose	20 g
Yeastrel	6 g
Tomato juice	200 ml
Water	to 1 liter

Tomato juice: Boil 1 pound tomatoes in 1 liter of water. Strain and discard pulp. Adjust to pH 7.2. Sterilize at 15 pounds pressure 20 minutes.

Assay procedures: Dilute cream 1 + 2 with N/50 HCl. Prepare nisin solution of 1000 units per ml by dissolving 0.1 g mega unit nisin in 100 ml N/50 HCl. Make further dilutions of nisin of 50, 40, 30, 20 and 10 units per ml. Melt agar and cool to 45 C. Using a culture of *Lactobacillus bulgaricus* not over 48 hours old seed plates of tomato dextrose agar with a 0.1 per cent inoculum of this organism. Approximately 0.1 per cent inoculum is made by diluting 1 ml of culture with 9 ml of milk and transferring 1 ml of resultant culture into a Petri plate to which about 12 ml agar is added. When the agar is set punch 4 holes in each plate with a size 8 cork borer. Add 0.15 ml of nisin solution of each concentration and the test solution to the respective holes in the plate. Store plates 18 hrs 4 C to allow nisin to diffuse through the agar. Incubate 24 hrs 37 C. Examine the zones and measure their diameter.

Reading result:

$$\frac{4 \times \text{diameter of zone}}{4} - \frac{4 \times \text{diameter of hole}}{4} = \text{Average zone of inhibition}$$

Plot zone of inhibition against concentration of nisin. Obtain nisin concentration in sample from result obtained for the test solution and adjust for the dilution.

SPECIAL SUPPLIES

Agglutinating and precipitating diagnostic sera

Wellcome Research Laboratories, Langley Court, Beckenham, Kent, England

Anaerobic jar with external indicator capsule. BTL modified McIntosh and Fildes pattern. Catalog Nr D6-572

Baird and Tatlock Ltd, Freshwater Road, Chadwell Heath, Essex, London, England

Bunsen burners with bypass and red and green control knobs

Arnold R. Horwell Ltd, 17 Cricklewood Broadway, London NW 2, England

Plate gauge single hole 0.95 mm diameter for making small bore Pasteur pipettes to deliver 50 drops per ml

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Esco Rubber Ltd, 34 Somerford Grove, London N 16 England

Recommendation: Implementation of a program of food inspection designed to detect food poisoning microorganisms according to the methods outlined in these procedures is recommended for the Defense Subsistence Supply Center Laboratory. Such a program would generate a wholesome effect on food processors and suppliers.

After the activated program is in operation, it is further recommended that Dr Betty Hobbs, Director of the Food Hygiene Laboratory in London, be invited to visit our laboratory. The objective would be to have her render a critique and appraisal of the program with the offer of advice and counsel in those areas where the program could be strengthened.

GPO 809-110-9

PART IV

REPORT OF FIELD TRIPS MADE
TO VISIT VARIOUS
AUTHORITIES, LABORATORIES AND RESEARCH CENTERS

during the course of a

Secretary of the Army's

Research and Study Fellowship

by

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Laboratory Division
Directorate of Technical Operations
Defense Subsistence Supply Center
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AUTHORITIES, LABORATORIES AND RESEARCH CENTERS VISITED

Summary: By far the great majority of experts specializing in the study of food poisoning by Clostridium perfringens are centered in Great Britain. Of the small minority conducting research on this subject in the United States, the Robert A. Taft Sanitary Engineering Center in Cincinnati, Ohio is one of the leaders. Contacts were made to visit as many research institutions active in this field as possible while I was stationed in their vicinity.

The organizations selected were in the process of studying the food poisoning characteristics of C perfringens from different points of view depending on their specific interest and research activity. Precise and detailed information was being developed on this subject in divers but related fields by public health laboratories, universities, food research institutes, medical research circles and agricultural research councils.

These public spirited organizations are generally liberal in their discussions, at least with authorized visitors, of research they are conducting. Specific information, if available, is gladly and freely given to visiting scientists with proper credentials. Such information critically analyzed and adequately considered in an overall study can materially enhance the technical value of a report. Furthermore, from the added information gained, a realistic evaluation could cancel fruitless plans and reveal research possibilities or avenues of endeavor hardly likely without the experience of a visit to such inspiring environments. Every effort was therefore made to schedule as many visits of this kind as was possible in an itinerary, and at the times mutually satisfactory to the visitor and those visited.

Highlights of educational value from each of the associations, where on an average a half day was spent, follows:

Place: Robert A. Taft Sanitary Engineering Center, United States Public Health Service, Department of Health, Education and Welfare, 4676 Columbia Parkway, Cincinnati 26, Ohio
Date: 29 May 1961
Persons: Dr. Robert Angelotti, Dr. Herbert E. Hall
Objective: Inquiry was made to obtain the details of a method of analysis, as yet unpublished, which was in the final stages of development by the authors. It was to be entitled: "A rapid procedure for the detection and quantitation of C perfringens in food."
Findings: The method depends upon the use of a modified iron sulfite agar in which sulfite reducing Clostridia grow luxuriantly and form black colonies. Polymixin and sulfadiazine are added to the medium to inhibit sulfite reducing species of other genera, resulting in a selective medium for Clostridia.

in foods. Black colony formation in this medium within 24 hours incubation in a 10 per cent CO_2 and 90 per cent N_2 anaerobic atmosphere provides presumptive evidence of the presence of Clostridia. Differentiation of C perfringens picked from black colonies that could be due to any of 93 species of Clostridia is accomplished by its characteristics of nitrate reduction, H_2S formation, and lack of motility, confirmed by spore formation in Ellner medium.

Conclusion: The method is suitable for detecting C perfringens but does not distinguish any of the types A, B, C, D, E or F nor the food poisoning strains.

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Place: Pfizer Limited, Sandwich, Kent, England

Date: 12 July 1961

Persons: Dr. O. Andria and Dr. C. A. E. Briggs

Objective: This leading pharmaceutical production firm was visited as part of the program arranged for members of the Society for Applied Bacteriology attending the summer conference at Wye College, Ashford, Kent, England.

Findings: Members had the opportunity of hearing a lecture on vaccine production and were taken on a tour of the vaccine unit. The itinerary included a showing of the fermentation area and a visit through the pharmaceutical manufacturing division and laboratories. Details of a paper on C perfringens in the pig, presented by the above-named authors at the Wye College conference, were obtained.

Conclusion: The excursion was well conceived and of educational value.

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Place: The Oxoid Division, Oxo Limited, Thames House, Queen Street Place, 16 Southwork Bridge Road, London, England

Date: 15 August 1961

Persons: Dr. J. Elstub, Mr. W. H. Pierce, Mr. R. F. Roberts and Mr. D. Huckerby

Objective: Observation of the process and consideration of the problems involved in the manufacture of microbiological culture media, ingredients and products used in laboratory procedures for detection, isolation and determinations of microorganisms was of interest to this Fellowship project.

Findings: A tour of the production plant revealed the steps in the manufacture of dehydrated and prepared culture media, ingredients and reagents used in the examination of food products and other substances; in the cultivation of specific types of microorganisms; and in the procedures for microbiological assays. Laboratories for the control of the purity of the raw material, the condition of the items in process and the quality of the finished products were also visited.

Conclusion: Oxoid is one of the leading producers of culture media and allied materials. The tour was of exceptional interest and value.

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Place: Medical Research Council, Industrial Injuries and Burns Unit, Birmingham Accident Hospital, Bath Row, Birmingham 15, England

Date: 29 August 1961

Person: Mr. H. A. Lilly

Objective: As an authority in culture methods for Clostridia, Mr. Lilly's views were sought concerning the sporulation characteristics of C perfringens. Production of spores of this organism under laboratory conditions is too often a trial and error process. The immediate problem on which we sought enlightenment was how to produce a considerable quantity of spores for study in food products at low temperatures.

Findings: In 1949, Dr. S. A. Waksman discovered that patients whose wounds were infected with C perfringens did not show any improvement when treated with the antibiotic neomycin. Noting this resistance of C perfringens to neomycin Mr. Lilly incorporated this antibiotic in a culture medium which was designed to inhibit other organisms and isolate C perfringens.

Conclusion: While the information desired on sporulation was not available it was advantageous to learn first hand how the idea of utilizing neomycin in the Nagler plate, as being selective for C perfringens, was developed.

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Place: Department of Bacteriology, The School of Medicine, University of Leeds, Leeds 2, England

Date: 30 August 1961

Person: Dr. A. Trevor Willis

Objective: Dr. Willis had recently published a textbook on anaerobic bacteriology and numerous papers on the subject in the past five years. In view of his status as a world authority on the anaerobes information was sought on certain technical difficulties in cultivating and isolating the Clostridia.

Findings: Certain points contained in the several papers and textbook by the author were emphasized. Anaerobes are not suitable as an index of fecal pollution of water because a high proportion of them, usually non-food-poisoning strains of C perfringens may be derived from the surrounding soil. Consequently the sulfite reduction test using Wilson-Blair medium is of doubtful value in assessing the fecal pollution of water. However, the test is of value in examining treated waters for the presence of C perfringens to determine the efficiency of the filtration process. These organisms, which are predominantly sporulated, survive chlorination with subsequent development of vegetative forms due to aftergrowth. Dr. Willis considers the Wilson-Blair sulfite reduction test unreliable for anaerobes since some species of Clostridia do not respond to it and under certain conditions false positive reactions are obtained. The author recommends his modified Nagler medium using egg yolk instead of human blood serum for detecting C perfringens. This organism is the only lactose fermenting Clostridium which produces lecithinase. Production by an anaerobe of opalescence in Dr. Willis' medium, which is

inhibited by antitoxin of C perfringens, together with formation of a red halo is diagnostic of this anaerobe. The red halo eliminates C bifermentans since the latter does not ferment lactose, but otherwise gives an opalescence similar to C perfringens. In a further study Dr. Willis showed that C perfringens does not contain a lipase but that its action on egg yolk is due to lecithinase. He uses streptomycin as an inhibitor of aerobic spore formers. In studying C perfringens occasional strains do not give a stormy fermentation with milk because they are lactose negative; Bacillaceae may interfere with C perfringens isolation since they are only partly inhibited by neomycin; and aerobes such as Bacillus cereus, can grow anaerobically, and may give a Nagler reaction that is not inhibited by antitoxin. One must be on the lookout for strains with such atypical characteristics.

Conclusion: The useful information generously furnished by Dr. Willis made this a most profitable and inspiring visit.

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Place: Department of Scientific and Industrial Research, Food Investigation Organization, Torry Research Station, 135 Abbey Road, Aberdeen, Scotland

Date: 31 August 1961

Persons: Dr. James M. Shewan and Dr. G. Hobbs

Objective: It was desirable to note the operations and main developments featured in this organization which is concerned principally with research on catching, handling and processing fish and other water foods.

Findings: A tour of the station's fishing vessel, Sir William Hardy, was arranged during which an explanation was given on landing fish, fish hold design, freezing methods and transport of fish. More rapid cooling of fish is accomplished by contact with water from melting ice than by the freezing action of commercial refrigerants. Dr. Shewan demonstrated and discussed storage conditions, use of flake ice and preservative ices, physical and biochemical changes during storage, air, water and dielectric thawing of fish, and quality and sensory assessment of frozen fish. Research in the preservation of fish for food by drying, salting, smoking and canning was considered. Research in the utilization and production of fish byproducts from surplus fish and fish waste, such as fertilizer and fish meal was pointed out. Finally the microbiology of fresh fish and of fish spoilage was reviewed.

Conclusion: The knowledge gained in this extremely interesting tour will be especially useful in discharging our responsibilities in the DSSC in the analysis of fish and water food products for contract requirements.

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Place: University of Edinburgh, Bacteriology Department, University New Buildings, Teviot Place, Edinburgh 8, Scotland

Date: 4 September 1961

Person: Dr. J. Gerald Collee

Objective: Dr. Collee presented results of research on food poisoning strains

of *C perfringens* at the July 1961 conference at Wye College, Ashford, Kent, England that he and his colleagues had recently concluded. Since the topic was of special interest to this fellowship project, whatever specific details concerning his observations that Dr. Collee could furnish prior to the publication of his paper were most welcome.

Findings: Characteristics of classical type A beta-hemolytic strains were contrasted with food poisoning strains of *C perfringens*. Vegetative cells of the former produce distinct zones of beta hemolysis on blood agar plates and the spores are not heat resistant. Food poisoning strains produce no hemolysis or at best weakly hemolytic alpha zones. Spores of these strains are capable of surviving the heat of boiling water for one to five or more hours. Both of these strains are present in the contents of human intestines. Dr. Collee found food poisoning strains in 39 of 50 fecal specimens examined, although only three of these were markedly heat resistant by the accepted criterion of survival after cooking in meat broth for one hour at 100 C. He observed that spores do not normally occur in significant numbers in cooked food. Heat resistance tests for spores revealed the protective effect of cooked meat. The heat resistant property was genetically determined and not dependent on the presence of a large number of spores.

Different strains vary, sometimes atypically, in the expected characteristics. A sporulating medium described by Ellner in 1956 produces variable spore yields from strains known to be spore formers. Serological identification by the procedure of Hobbs' group described in 1953 is not always possible because although approximately 75 per cent of strains may be serologically typed, untypable strains occur and cross reactions are encountered. An unusual growth pattern at 50 C which Dr. Collee named the "Phoenix Phenomenon", was observed with both heat sensitive and heat resistant strains. There was no difference between the growth curves of food poisoning and classical type A beta hemolytic strains. Both could grow under nearly similar conditions, which explains occasional outbreaks from classical type A beta hemolytic strains. Dr. Collee further explained that these strains were unlikely to survive cooking but may be introduced during the cooling period and be protected by lack of heat penetration. It is unlikely that all nonhemolytic strains have heat resistant spores; and it is doubtful that all heat resistant strains can cause food poisoning since considerable heat resistance of spores from both hemolytic and nonhemolytic strains has been demonstrated. It has been suggested that perhaps any strain of *C perfringens* under optimum conditions can cause food poisoning. This speculation is based on the observations of McKillop, who reported food poisoning caused by beta-hemolytic and non-hemolytic strains of *C perfringens* which contaminated chickens during the cooling period. The spores, and possibly vegetative cells were present in the metal container into which the chickens and liquor were transferred after cooking. Nevertheless, the great majority of food poisoning oubreaks due to *C perfringens* are

caused by heat resistant nonhemolytic strains. These two characteristics and serological and toxicological typing are important in differentiating typical food poisoning strains of this organism from non-food-poisoning strains.
Conclusion: Altertness for observation of possible anomalies in the characteristics of C perfringens was the keynote of Dr. Collee's experience with this organism.

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Place: University and Western Infirmary of Glasgow, Department of Bacteriology, Bearsden, Glasgow, Scotland
Date: 5 September 1961
Persons: Dr. J. W. Howie, Dr. W. L. Weipers and Dr. E. M. Harper
Objective: As chairman of the Department of Bacteriology Dr. Howie had directed research on a number of practical projects concerned with outbreaks of food poisoning. An audience with him and his associates for their viewpoint on the subject was highly desirable.
Findings: Dr. Howie had conducted a considerable number of studies feeding animals food contaminated with C perfringens. He concluded that domestic and experimental animals are immune to the strains known to cause human food poisoning, for, these strains have so far been found to be nonpathogenic for animals. He declared that it was not certain how this organism acts to affect humans. Liberation of an endotoxin within the intestine, or release of a specific enterotoxin, or production of a nonspecific toxic breakdown product from the food were possibilities. Since the incubation period is 8 to 18 hours the organism apparently establishes an infection within the intestine. As a normal inhabitant of soil and intestinal contents of animals and man it is widely distributed in water, dust, insects, food and sewage. Contaminated meats that have been cooked on one day and eaten the next are the greatest cause of trouble. Heat resistant spores survive the cooking and germinate and multiply rapidly during cooling. Survival of spores is favored when the food is cooked in large portions because, as indicated by Miller, meat is a poor conductor of heat. Growth is also enhanced because air cannot penetrate into these bulky portions, thus providing the required anaerobic conditions for spores. Cooking drives off oxygen and destroys other organisms most of which cannot survive the heat tolerated by C perfringens. Ideal conditions for multiplication result due to lack of competition and to the rich nutrients present in meat. Meat should therefore be divided and cooked in small portions to ensure heat penetration to the center. Short periods of pressure cooking are more effective than longer periods of roasting, stewing or boiling. If the meat must be cooked the day before it is eaten it should be cooled rapidly to prevent germination of spores and reheated sufficiently to ensure destruction of any survivors. Food handlers should furthermore be cautioned of the possibility of contamination of the food after it is cooked. This is pointed out in a study conducted by Dr. Elizabeth J. McKillop in 1959 which Dr. Howie directed. The study revealed numerous instances of contaminated cooked food which was free of bacteria in

GPO 809-110-6

the raw state but caused food poisoning after contamination in the cooked state. Beta hemolytic, heat sensitive strains of _C perfringens_ which are involved under these conditions are not apt to cause food poisoning if the food is contaminated before cooking because they do not survive the heat of thorough cooking.

Conclusion: Personal contact with this authority on food poisoning and with his associates proved to be very profitable due to the realistic nature and usefulness of the findings of this research group.

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Place: Glaxo Laboratories Limited, Greenford, Middlesex, England

Date: 11 September 1961

Person: Dr. J. E. Crofts

Objective: During his research fellowship period at the University of Manchester Dr. Crofts had thoroughly investigated the action of penicillin on _C perfringens_ Type A. The possible use of pencillin as a therapeutic agent in cases of food poisoning by this organism could authoritatively be evaluated by Dr. Crofts.

Findings: Morphological changes of microorganisms _in vivo_ during the treatment of patients with penicillin have been known since Florey observed this phenomenon in 1949. Since _C perfringens_ is susceptible to the bactericidal effect of penicillin in patients Dr. Crofts investigated whether such changes are associated with a reduction of the virulence of the organism or with an increase in the defense mechanism of the body. He discovered that bactericidal concentrations of penicillin interfered with normal cell division of the organism. Elongated forms at least 100 times the length of normal cells were produced. Few or no transverse septa could be seen in these long bacilli. The cells tended to lose their Gram positive property and, in sufficiently high concentrations of the antibiotic, lysis occurred. Just before lysis however the cells lost their viability.

Neither the growth rate nor the production of alpha toxin, theta hemolysin or hyaluronidase was affected. The long forms reverted to normal in medium free from penicillin in a relatively short time. With the long forms the lag phase was slightly longer than normal but the generation time, maximum growth and alpha toxin production were similar to that with the normal forms. Long forms were almost as virulent in guinea pigs as normal bacilli and the latter changed to long forms when the animals were treated with penicillin. These changes did not affect the property of the organism to cause infections.

Conclusion: In order to protect against infection by _C perfringens_ the concentration of penicillin must be maintained at a bactericidal level and not merely sufficient to inhibit cell division.

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Place: Microbiology Division, Standards Department, Boots Pure Drug Co Ltd, Station Street, Nottingham, England

Date: 15 November 1961

Persons: Mr. G. Sykes and Mr. A. Royce

Objective: As president of the British Society for Applied Bacteriology and author of numerous papers and the popular textbook "Disinfection and Sterilization" it was considered opportune and educational to visit Mr. Sykes and the organization he directs.

Findings: Besides arranging an interesting tour of the Microbiology Division, Mr. Sykes furnished copies of the following procedures:

1. A general method for turbidimetric or titrimetric microbiological assay of vitamins.
2. A general method for the microbiological assay of vitamins and antibiotics by the plate diffusion method.
3. Method for the assay of thiamine.
4. Two schemes for a Latin square design for the diffusion assay method using the large plates.

Conclusion: The procedures furnished are time saving devices that will be useful on occasions when great numbers of samples requiring the same test to be performed are received.

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Place: Food Research Department, Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford, England

Date: 22 November 1961

Persons: Dr. A. C. Baird-Parker, Dr. G. W. Gould, Dr. A. D. Tadd, Dr. John Cavett, Dr. M. B. Gibbs, Dr. A. Hurst and Miss B. Freame

Objective: Dr. Baird-Parker, Dr. Gould and Dr. Tadd each presented a paper on food microbiology at the summer conference of the Society for Applied Bacteriology at Wye College, Ashford, Kent, England. Dr. Gibbs and Dr. Hurst developed the reinforced *Clostridial* medium commonly used in diagnostic laboratories for the isolation of these anaerobes. Dr. Hurst also presented a paper on disinfection before the S A B at a conference held a few weeks previously at Queen Elizabeth College, London. Dr. Cavett had a paper in press on the microbiology of bacon. It was therefore considered apropos of this project to visit these authorities in food microbiology research while stationed in the vicinity of their headquarters.

Findings: A tour of the pilot plant and research facilities was arranged. About a half hour was devoted to conferring with each of the section chiefs. Details of an improved selective and diagnostic medium for isolating and enumerating coagulase positive *Staphylococci* in frozen foods was obtained from Dr. Baird-Parker.

Conclusion: Exchanging viewpoints and ideas on problems of mutual interest with the principal scientists of this active food research organization was a profitable experience.

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Place: The British Food Manufacturing Industries Research Association, Randalls Road, Leatherhead, Surrey, England

Date: 28 November 1961

Persons: Mr. J. C. Dakin and Mr. H. L. Shipp

Objective: At the suggestion of Dr. M. Ingram, sponsor of the phase of this fellowship project conducted in England, a visit of the laboratories of this association was undertaken to observe the functioning of a unique research organization financed by the pooled monies of the food producers of Great Britain. Mr. Dakin's research was concerned with microbial survivors in food; and Mr. Shipp's with food poisoning microorganisms.

Findings: A comprehensive tour of the several research departments revealed a broad array of investigations which are conducted in the physical, biochemical and microbiological sciences to resolve the more practical problems in food technology with which the industry is concerned.

Conclusion: Educational and practical benefits were derived as a result of viewing these efficient research facilities.

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Place: The Wellcome Research Laboratories, Langely Court, Beckenham, Kent, England

Date: 8 December 1961

Persons: Dr. G. Harriett Warrack, Dr. F. V. Linggood and Dr. M. Sterne

Objective: In collaboration principally with Dr. C. L. Oakley, Dr. Warrack had published numerous papers on the Clostridia and their toxins, especially that of C perfringens. Her papers on the toxicological typing of C perfringens were of particular value to this fellowship study. A personal visit of her facilities was deemed to be of prime importance.

Findings: Discussion of the complex problems present in the study of the toxins of C perfringens was followed by a tour of the production plant. In the Wellcome Research Laboratories, antisera, toxoids, vaccines and tuberculins are produced for world wide distribution. Dr. F. V. Linggood, an authority on diphtheria toxin and antitoxin, pointed out the steps in the large scale production of diphtheria toxoid and antitoxin as typical of the production of other antisera in great quantities. The immunized horses from which antitoxin serum is withdrawn were examined. Concentration and purification of the antitoxic serum by precipitation of undesired proteins and by dialysis was observed. Preparation of certain antiviral vaccines by inoculation of attenuated strains of virus into fertile hens eggs and extraction from the embryro was also noted. Testing of the end product for potency, sterility and absence of foreign toxic materials was demonstrated. Similar to the production of diphtheria antitoxin were antibacterial sera, antiviral sera and antivenom products.

Conclusion: Observation of the processes involved in the production of antigens and their corresponding antibodies for prophylaxis and treatment, and the procedures and precautions required in their storage, handling and

distribution was of considerable educational value. Emphasis of special points dealing with the toxicological typing of *C perfringens,* based on Dr. Warrack's two papers specifically concerned with this subject, was of immediate usefulness to the project.

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Place: St. George's Hospital, Hyde Park Corner, London, England
Date: 13 December 1961
Persons: Dr. F. E. Dische and Dr. S. D. Elek
Objective: Of all projects conducted in the study of experimental food poisoning by *C perfringens* on human volunteers the investigation undertaken by Dr. Dische and Dr. Elek was the most thorough and extensive. This visit afforded an opportunity for authoritative inquiry into a type of research that can be only rarely undertaken since human subjects are involved.
Findings: Cultures of heat resistant type A *C perfringens* in Robertson cooked meat medium produced a short attack of abdominal pain and diarrhea after 12 hours in most of the 41 volunteers. Symptoms were the same as those occurring in reported food poisoning outbreaks. Negative findings occurred with cultures killed by boiling and with unheated filtrates free of the organism. Experiments with live cultures maintained in the laboratory were largely negative, indicating that virulence may be lost *in vitro.*

Apparently illness is due to an infection caused by the live food poisoning strains only. Alpha toxin was unlikely to be concerned in the disease because an antibody to this or any other toxin could not be found in the affected subjects. Consequently an attack did not confer protection against a further attack.
Conclusion: Prevention is possible by cooking meats properly and consuming them shortly thereafter. The danger of precooking meats and allowing them to cool slowly and then serving them some hours later or the next day upon superficial reheating was emphasized. If precooking is unavoidable, rapid cooling and refrigeration should be mandatory. Adequate reheating would make the food safe for consumption.

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Place: Lyons Laboratories, 149 Hammersmith Road, Cadby Hall, London W 14, England
Date: 20 December 1961
Person: Mr. D. H. F. Clayson
Objective: Dr. Ella Barnes, direct supervisor of the part of this Fellowship project conducted in Cambridge felt that it would be timely to see the laboratories of J. Lyons Limited since this company is a leading food producer and restaurant chain in London. The fact that Dr. Barnes worked in these laboratories in her early career facilitated the contact.
Findings: Mr. Clayson, a specialist in the hygiene of preparation and service of food and its perishability due to microbial spoilage, discussed the biochemical changes in food constituents resulting from enzymatic action of

pertinent microorganisms. For example, collagenase from C histolyticum attacks gel-containing products with gas formation. In products such as custard pies, syneresis, which causes exudation of water from gels as they age, accelerates spoilage. He mentioned a recent process whereby meat is tenderized through antemortem vascular injection of proteolytic enzymes. The visit included a tour of the analytical and research laboratories and parts of the food production plant where ice cream bars, meat pies and food specialty items are made.

Conclusion: It was a revealing tour with a considerable number of theoretical and practical concepts featured stressing prevention of spoilage and food poisoning through sanitation and hygiene.

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Place: The Lister Institute of Preventive Medicine, Bacteriology Department, Chelsea Bridge Road, London, England

Date: 20 December 1961

Person: Dr. Marjorie G. Macfarlane

Objective: Dr. Macfarlane had published numerous papers on the biochemistry of the bacterial toxins, the majority of them as the sole author. A visit with her was desired in order to acquire a better understanding of some of the more complex phases of her fundamental research.

Findings: In discussing the biochemistry of the toxins of C perfringens Dr. Macfarlane pointed out that this organism attacks histidine. One of the products of the reaction is histamine which may or may not be associated with food poisoning. Histidine decarboxylase plays a role in this reaction.

The alpha toxin, or lecithinase, is hemolytic in vitro in the presence of calcium ions. Phosphate ions are not required. This toxin is lethal upon injection into guinea pigs. The theta toxin of C perfringens is beta hemolytic. Sulfhydryl groups supplied by cysteine or thioglycollate activate the enzymatic hemolysis. Theta toxin is oxygen labile. A pharmacological reaction may be effected by putrescine which may be formed from ornithine; or by cadaverine which may be formed from lysine. Synergistic effects may be responsible since organisms common in the intestinal tract such as Escherichia coli play a role in converting these pertinent amino acids into the strongly basic and possibly toxic amines.

Conclusion: Dr. Macfarlane clarified certain intricate biochemical concepts stemming from the complex nature of the toxins of C perfringens. She conveyed the impression that the subject was really not well understood even by experts specializing in this field since several controversial findings have been reported.

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Place: Office of Naval Research, 429 Oxford Street, London W 1 England

Date: 2 January 1962

Person: Dr. Jackson W. Foster

Objective: Dr. Foster was currently on a research fellowship at this station from his home assignment as a professor in the Department of Bacteriology, University of Texas, Austin, Texas. He is known for his authoritative studies and publications on sporulation and germination of bacterial spores. A visit with him was considered to be beneficial in connection with the study of the characteristics of the spores of C perfringens.

Findings: Discussion concerning Dr. Foster's research brought out several salient points. Besides the full genetic complement of a bacterium the endospore contains certain characteristic chemical constituents. Among these, dipicolinic acid predominates. These constituents are present in a spore coat made up of unique proteins which are unrelated to the proteins present in the vegetative cell. Mineral deficiencies in complex organic media are limiting factors in the sporulation of bacilli since certain inorganic constituents, namely manganese, potassium, magnesium, cobalt iron, zinc, phosphorous and sulfur are variously essential for sporogenesis of the different organisms. Likewise, specific organic nutrients function as biochemical determinants for sporulation and spore germination. Dr. Foster proposes that spore formation comprises a rearrangement of materials already present in the vegetative cell. Under appropriate conditions these cells can be irreversibly committed to sporogenesis. Eventually there is practically complete conversion to spores. The phenomenon of sporulation transpires during a strikingly short time interval. In certain experiments with an ionic surfactant, spores can be germinated instantaneously. Less than five seconds is required for the transition from spore to vegetative cell. In this brief period obligate aerobes will germinate anaerobically. Spores can be germinated mechanically with scratches of small dimension--of the order of 40 microns--on ground glass. From these observations Dr. Foster concludes that spore germination is not a metabolic process.

Conclusion: Rather than being concerned with practical matters about sterilization or the time and temperature required to kill spores, or the sporicidal effect of radiation, Dr. Foster offers ideas and research on the basic physiology of these hardy entities. This wholesome approach should contribute to the accruing momentum for research to provide an insight into the fundamental mechanisms of sporulation and spore germination. Practical results would more rapidly follow a fundamental understanding of these basic mechanisms.

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Place: National Institute for Research in Dairying, University of Reading, Shinfield, Berkshire, England

Date: 15 January 1962

Persons: Dr. J. G. Davis, Dr. R. G. Baskett and Dr. J. E. Ford

Objective: At the recommendation of Dr. Betty C. Hobbs, sponsor of the portion of this fellowship project conducted in the Food Hygiene Laboratory, Colindale, London, this field trip was undertaken for its educational value.

Findings: This institute is State financed through the Agricultural Research Council. Fundamental research is carried out to secure a better understanding of the physiology of milk production, the nutrient requirements of dairy cattle and the physical, biochemical and microbiological significance of the changes involved in the processing of milk and its products. The Institute comprises ten research departments, namely, Dairy Husbandry, Feeding and Metabolism, Physiology, Radiobiochemistry, Bacteriology, Chemical Microbiology, Chemistry, Physics, Engineering and Nutrition. About 400 persons number the total staff of which some 120 are professional personnel. The eagerness and intensity of investigative work is immediately evident. A summary idea may be gleaned of the prolific amount of research accomplished by these ten departments from a consideration of the 165 publications appearing in various technical journals over the course of only one year, from October 1959 to September 1960. All of the titles of these papers, as well as a synopsis of recent research findings, and projects under way in each of these ten departments appear in the comprehensive annual report for 1960 of this Institute which is listed in the literature cited.

Conclusion: The overwhelming amount of high caliber research accomplished by this active organization speaks well for its progressive management and serves as an inspiration to visitors engaged in any field of scientific endeavor. This activity was most impressive and gave every evidence of careful design and construction with flexibility of use of the facilities.

Recommendation: During the course of this Fellowship study, overseas visits to the following three research institutes on the continent were considered essential:

Dr. E. H. Kampelmacher, Rijs Instituut voor de volksgezondheid, Sterrenbos 1, Utrecht, The Netherlands

Dr. R. Buttiaux, Centre d'Enseignement et de Recherches, de Bacteriologie Alimentaire, de l'Institut Pasteur de Lille, Lille, France

Dr. D. A. A. Mossel, Institut Central de la Nutrition, et de l'Alimentation T N O, Utrecht, Pays-Bas, The Netherlands

These visits could not be managed because the extra time required for this purpose would have extended the Fellowship period about 30 days beyond the scheduled expiration date. A one-week consultation period was to be spent with Dr. Kampelmacher followed by two weeks of indoctrination in Dr. Mossel's laboratory. This was to be terminated by one week's guidance with Dr. Buttiaux.

Such field trips as described above have proven to be a very useful and practical part of this Fellowship study. Experiments planned, or under way in

these institutes have a promise of untold value to our interests. It is good to be guided by the best talents in this work. For this reason, it is recommended that the options to accept these invitations to study the latest approaches to the solution of food poisoning problems with these world authorities be favorably considered.

In the United States a research group at the University of Wisconsin, led by Dr. D. H. Strong recently reported results of detection and enumeration of <u>C perfringens</u> in foods. It would be well to visit this department also.

GPO 809-110-7

PART V

SCIENTIFIC MEETINGS ATTENDED

during the course of a

Secretary of the Army's

Research and Study Fellowship

by

John E. Despaul, Assistant Chief
Laboratory Division
Directorate of Technical Operations
Defense Subsistence Supply Center
1819 West Pershing Road
Chicago 9, Illinois

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SCIENTIFIC MEETINGS ATTENDED

Demonstrations at the Medical Research
Council for the Ministry of Health
Public Health Laboratory Service
Colindale
London, England
30 June 1961

Author	Topic	Laboratory Location
G V Helmer and C E D Taylor	A fluorescent antibody technique	Colindale
P Chadwick	Rapid identification of bacteria by fluorescent antibody	Birmingham
J E Jameson	A home made slit sampler	Brighton
M H Hughes	Drinking straws for pipettes	Winchester
J M B Edwards	Detection of fimbriae in Salmonella and Shigella organisms	Colindale
K Steel	Oxidative versus fermentative carbohydrate metabolism	Colindale
J G Wallace and J E M Whitehead	Experience with the disc method of testing sensitivity of tubercle bacilli to antibiotics	Coventry
D R Gamble	Coxsackie viruses	Epsom
J H McCoy	Characteristics of Salmonella colonies on bismuth sulphite medium	Hull
J C Kelsey	Ethylene oxide sterilizer - hospital pattern	Colindale
O M Lidwell and K P Carpenter	Apparatus for streaking nine cultures simultaneously	Colindale
M P Jevons	A modified technique of Staphylococcus phage typing	Colindale
D Kingston and W C Noble	The laboratory testing of bactericidal paints and other surface treatments	Colindale

Society for Applied Bacteriology

Conference at Wye College
Ashford, Kent, England
11 thru 13 July 1961

Author	Topic	Organization
G W Gould	Preliminary microscopic observations on spore germination in the genus *Bacillus*	Food Research Dept Unilever Ltd Sharnbrook
P D Walker and J Wolf	Some properties of aerobic thermophiles growing at 65 C	Dept of Agriculture Leeds University
A C Baird-Parker	A new differential and diagnostic medium for *Staphylococcus aureus*	Food Research Dept Unilever Ltd Sharnbrook
A C Hayward	Geographical variation in *Pseudomonas solanacearum*	Commonwealth Mycological Institute Kew
A J Holding and J King	The effectiveness of white clover rhizobia isolated from hill soils	Edinburgh School of Agriculture and Hill Farming Research Organisation Edinburgh
L A Allen	Production and characteristics of autolysates from food yeasts	Orpington
D H Shrimpton	The production of hydrogen sulphide by washed suspensions of *Escherichia coli*	Low Temperature Research Station Cambridge
A D Tadd and A Hurst	The effect of feeding colicinogenic *E coli* on intestinal *E coli* in early weaned pigs	Food Research Dept Unilever Ltd Sharnbrook
A H Emslie-Smith	Observations on the secular succession of types of *E coli* and related organisms in the faecal flora of an adult human subject	Dept of Bacteriology University of Durham
O Andrla and C A E Briggs	The incidence of lecithinase-positive *Clostridium welchii* in the pig	Veterinary and Agricultural Research Dept Pfizer Ltd Sandwich

Society for Applied Bacteriology

Conference at Wye College
Ashford, Kent, England
11 thru 13 July 1961

Author	Topic	Organization
E M Harper and W L Weipers	The gastrointestinal flora of the dog	Bacteriology Dept University of Glasgow
R Spencer	The occurrence of luminous bacteria in the intestinal tract of marine fish	D S I R Humber Laboratory Kingston-upon-Hull
M A Long	Some functions and malfunctions of microorganisms in the human gut	Metabolic Research Unit Little Bromwich Hospital Birmingham
H W Smith	The bacterial flora of the faeces of animals with particular reference to its development in the young	The Animal Health Trust Farm Livestock Research Centre Stock
H Haenel	Microecological rules in the composition of the intestinal flora of man	Institut fur Ernährung Potsdam-Rehbrücke
A B Dickinson and G Mocquot	Studies on the bacterial flora in the alimentary tract of pigs 1. Enterobacteriaceae and other Gram negative bacteria	Station Centrale de Recherches Laitières et de Technologie des Produits Animaux Jouy-en-Josas
P Raibaud, M Caulet, J V Galpin and G Mocquot	Studies on the bacterial flora in the alimentary tract of pigs 2. Streptococci: selective enumeration and differentiation of the dominant groups	Station Centrale de Recherches Laitières et de Technologie des Produits Animaux Jouy-en-Josas
M Lev	Germ-free animals and their use in elucidating the action of the gut flora on the host	National Institute for Research in Dairying Reading
J Taylor	Host specificity and enteropathogenicity of Escherichia coli	Central Public Health Laboratory Colindale

Society for Applied Bacteriology

Conference at Wye College
Ashford, Kent, England
11 thru 13 July 1961

Author	Topic	Organization
J G Collee J A Knowlden and B C Hobbs	Studies on the growth sporulation and carriage of *Clostridium welchii* with special reference to food poisoning strains	Bacteriology Dept Edinburgh and Central Public Health Laboratory Colindale
B C Hobbs	The public health significance of *Salmonella* carriers in livestock and birds	Central Public Health Laboratory Colindale
R Buttiaux and D A A Mossel	The significance of organisms of supposed faecal origin in foods and water	Institut Pasteur de Lille

Society for General Microbiology

Conference at University of Oxford
Oxford, England
28 thru 30 September 1961

(Lectures and Demonstrations)

Author	Topic	Organization
M R J Salton	Cell-wall structure and biosynthesis	Department of Bacteriology University of Manchester
P Mitchell	Metabolism transport and morphogenesis: which drives which?	Zoology Department University of Edinburgh
D E Hughes	The bacterial protoplast membrane	Department of Biochemistry University of Oxford
J Lascelles	The chromatophores of photosynthetic bacteria	Department of Biochemistry University of Oxford
K McQuillen	Bacterial ribosomes and protein synthesis	Department of Biochemistry University of Cambridge
S Dagley	Some effects of drugs and inorganic ions on bacterial ribonucleoprotein	Department of Biochemistry University of Leeds
G D Hunter	The final stages of protein synthesis and the role of lipids in the process	The A R C Field Station Compton nr Newbury Berks
A M Glauert and D A Hopwood	The fine structure of bacteria	Strangeways Research Laboratory and Botany School University of Cambridge
D H L Bishop and H K King	Intracellular distribution of Ubiquinone and vitamin K in bacteria	Department of Biochemistry University of Liverpool

Society for General Microbiology

Conference at University of Oxford
Oxford, England
28 thru 30 September 1961

(Lectures and Demonstrations)

Author	Topic	Organization
G L Jones D Woolley and F C Happold	Some metabolic differences between *T thioparus* *T denitrificans* and *T thiocyanoxidans*	Department of Biochemistry University of Leeds
B A Fry and J Sykes	The effect of induction of phage development on the ultra-centrifuge pattern of extracts of *Escherichia coli*	Departments of Microbiology and Biochemistry University of Sheffield
M Kramer V Csanyi and R Agfalvi	The influence of environment on penicillinase synthesis in the absence of specific inducer	Institute of Medical Chemistry Budapest
G W Gould	A spore enzyme produced by some strains of *Bacillus* capable of inactivating nisin	Unilever Research Laboratory Bedford
P E Reynolds	The mode of action of vancomycin	M R C Unit for Chemical Microbiology Department of Biochemistry University of Cambridge
A Hurst	The behaviour of some bacteria at high population densities	Unilever Research Laboratory Bedford
R J W Rees and E W Garbutt	Studies on tissue-culture-grown *Mycobacterium lepraemurium*	National Institute for Medical Research Mill Hill

GPO 809-110-6

Society for General Microbiology

Conference at University of Oxford
Oxford, England
28 thru 30 September 1961

(Lectures and Demonstrations)

Author	Topic	Organization
A Mayr-Harting	Some properties of colicine receptors	Department of Bacteriology University of Bristol
J F Watkins	Effects of intranasal inoculation of adult white mice with trachoma virus (T'ang strain)	Sir William Dunn School of Pathology University of Oxford
I Dickinson	The isolation of the virus of contagious conjunctivo-keratitis of sheep	Research Department Boots Pure Drug Co Ltd Nottingham
N R Grist	The haemagglutinin of Coxsackie A7	Department of Virology University of Glasgow
P de Somer and M C Vandeputte	Polyoma virus synthesis in rat tumour cells	Rega Instituut Leuven Belgium
J G Carr	The haemorrhagic disease induced by some fowl tumour viruses	British Empire Cancer Campaign Poultry Research Centre Edinburgh
G C Ainsworth	Can cultures be used as stable types?	Microbial Systematics Group
R Dougherty and P Simons	Antigenic variants of Rous sarcoma virus	Imperial Cancer Research Fund Mill Hill
L V Crawford	Viral contamination of tumour viruses	Institute of Virology University of Glasgow

Society for General Microbiology

Conference at University of Oxford
Oxford, England
28 thru 30 September 1961

(Lectures and Demonstrations)

Author	Topic	Organization
M H Salaman, K E K Rowson and J J Harvey	Studies on experimental and natural transmission of Moloney's leukaemogenic virus	The London Hospital
J F A P Miller	Studies on the gross leukaemia agent	Pollards Wood Research Station Bucks
K E K Rowson, D H Adams and M H Salaman	An enzyme modifying virus and its relation to mouse tumours and mouse leukaemias of various types	The London Hospital
G Klein	Virus-cell relationships in polyoma infected normal and neoplastic cells	Department of Tumour Viruses Karolinska Institute Stockholm
G Negroni and F C Chesterman	Virus-cell relationships in tumours induced by the Mill Hill polyoma virus	Imperial Cancer Research Fund Mill Hill
M G P Stoker and I A Macpherson	The *in vitro* transformation of hamster cells by polyoma virus	Institute of Virology University of Glasgow

Society for Applied Bacteriology

Conference at Queen Elizabeth College
Campden Hill Road, Kensington
London, England
24 October 1961

(Lecture and Demonstrations)

Author	Topic	Organization
G Sykes	The philosophy of disinfectant and antiseptic evaluation	Standards Department, Boots Pure Drug Co Ltd Nottingham
D Kingston and W C Noble	The testing of surfaces for bacteriocidal action	Central Public Health Laboratory Colindale
A H Walters and T G Mitchell	A comparison of two methods of evaluating sanitizers with special reference to a source of variation from neutralization	Diversey U K Ltd London
A Hurst	Assessment of the effect of disinfectants and cleaning agents on bacteria deposited on surfaces	Unilever Research Laboratory Colworth House Sharnbrook
R C S Woodroffe	A quantitative method for estimating the activity of germicides on skin	Unilever Research Laboratory Colworth House Sharnbrook
R M Fry	A method for testing the efficiency of formaldehyde as a sterilizing agent for Staphylococci	Public Health Laboratory Cambridge
D V Carter	The quantitative assessment of antibacterials and enzymes by the diffusion plate-assay technique	Standards Department Boots Pure Drug Co Ltd Nottingham
A M Cook	The Berry method for determining mean single survivor time of "Escherichia coli" in a disinfectant	London School of Pharmacy
A F Hams	From laboratory to field--a study in correlation	Research Division Boots Pure Drug Co Ltd Nottingham

Society for Applied Bacteriology

Conference at Queen Elizabeth College
Campden Hill Road, Kensington
London, England
24 October 1961

(Lecture and Demonstrations)

Author	Topic	Organization
B M Freeman	Ionizing radiation used for the elimination of Salmonellae in foods	Wantage Research Laboratory A E R E
A M Glauert assisted by R A Parker	Sectioning bacteria for electron microscopy	Strangeways Research Laboratory Cambridge
W K Smith	Rotating soil percolator	I C I Ltd Welwyn
J F Marten	Automated microbiological analysis	Technicon Instruments Co Ltd
A C Baird-Parker	An improved selective and diagnostic medium for isolating coagulase positive Staphylococci	Unilever Research Laboratory Colworth House Sharnbrook
R Spencer	Luminous bacteria	D S I R Hull

Society for Applied Bacteriology

Conference at Royal Society of Medicine
London, England
10 January 1962

Author	Topic	Organization
S B Thomas	Some contributions on the history of the Society	N A A S Trascoed Aberystwyth
J McCoy	The isolation of Salmonellae	Public Health Laboratory Kingston-upon-Hull
M J Thornley	The relative resistance to ionizing radiation of strains of Pseudomonas and Achromobacter	Low Temperature Research Station Cambridge
J G Davis and G A Howe	An investigation of iodophors in dairy practice	J G Davis & Partners
J G Davis	The testing of detergent sanitizers	J G Davis & Partners
L B Quesnel	The development of bacterial clones in a bacteriofrenic environment	Dept of Bacteriology University of Bristol
R S Hannan	Some properties of food packaging materials which relate to the microbial flora of the contents	T Wall & Sons (Meat & Handy Foods) Ltd Willesden London
M Ingram	Microbiological principles in pre-packaging meats and poultry	Low Temperature Research Station Cambridge
J J Cavett	The microbiology of vacuum packed sliced bacon	Unilever Ltd Food Research Lab Colworth House Sharnbrook Bedford
R G Tomkins	The conditions produced in film packages by fresh fruit and vegetables and the effect of these conditions on storage life	Ditton Laboratory Larkfield Maidstone Kent

REVIEW OF RECOMMENDATIONS

A total of 26,886 cases of "Gastro-Enteritis, Food Poisoning, and Dysentery" was reported in the United States to the Public Health Service in 1961. This was tabulated in the Annual Supplement of Morbidity and Mortality. These are optionally reported diseases listed by individual states. From the data presented it appears likely that much less than 10 per cent of the total incidence of these diseases is reported at all. This is evident in the fact that 32 of the states, and the District of Columbia made no report whatever. For the 18 states reporting, wide extremes are shown with lows of 3 cases each for New Jersey and Wisconsin, and an outstanding high of 13,028 cases for sparsely populated New Mexico. Alaska showed 493 cases, Hawaii 1,456. The remaining 13 states ranged from 14 for Nevada to 3,793 for Arizona.

It is of interest to speculate what proportion of these cases was due to food poisoning by Clostridium perfringens. No attempt was made in this listing to separate the non-specific diarrheas and gastroenteritis from food poisoning per se. For example, of the 13,028 cases reported from New Mexico 3 were designated food poisoning and the rest fall into the unspecified category.

In order to supplement these statistics Dr. Del W Ruthig of the Communicable Disease Center in Atlanta, Georgia, where this report originates, furnished additional information. From separate official reports of food poisoning from the states, 190 outbreaks were mentioned of which 7 were due to C perfringens. These accounted for 599 individual cases of C perfringens food poisoning among 6,955 persons afflicted with this disease during 1961. However, the type of food poisoning contracted by 2,763 of these persons fell into the category of unidentified etiology. Many of these undesignated cases could be due to this organism since failure to use anaerobic culture techniques may have caused outbreaks, suggestive of C perfringens, to go unnoticed.

In California, an increasing incidence of foodborne disease has been attributed to C perfringens. A fourfold increase in reported cases in 1960 over 1959, and another fourfold increase in 1961 over 1960 emphasizes the great effort being applied in California compared with other states in attempts to reckon with this disease. This serves as an index of the existing margin for improvement elsewhere. Even so, 50 per cent of reported foodborne outbreaks in California are categorized as "etiology unknown."

Advancement of knowledge in this field through further research and training would generate an increased interest in the subject, and make better control measures possible. Recommendations to that end have been made in the first four parts of this report. These are briefly restated here to serve as reminders of

the suggestions previously mentioned. In summary, the following recommendations are reiterated:

1. Prevent food poisoning by cooking foods so that heat resistant spores are destroyed either by steaming under pressure, thorough roasting, or frying or grilling. Cooking foods at boiling temperature will not destroy spores of heat resistant strains. Therefore, when meat is boiled, care should be taken to see that it is served hot immediately. Otherwise, it should be cooled rapidly and placed in a refrigerator within one hour of cooking and not exposed thereafter to warm temperatures. Further precautions to practice in order to prevent food poisoning consist of serving foods decidedly hot or cold, refrigerating immediately for later use, reheating leftovers thoroughly, and roasting meat in small chunks as outlined in the FOREWORD.

2. Extend the studies reported in Part I to include more nitrogen compounds and additional species of Clostridia to make the scheme of analysis more selective for Clostridium perfringens.

3. Develop a method for producing copious amounts of spores of C perfringens in order to augment the work reported in Part II. More spores would facilitate additional germination, heat survival, growth, and serological studies.

4. Activate a food hygiene section in the Laboratory Division, Directorate of Technical Operations, Headquarters, Defense Subsistence Supply Center, to examine foods for the possible presence of food poisoning microorganisms according to the procedures reported in Part III. Later invite Dr. Betty Hobbs to evaluate the program.

5. Authorize visits to two world renowned laboratories in Utrecht, The Netherlands, and one in Lille, France to complete the program of visitation recommended by Dr. Betty Hobbs while I was stationed in the Food Hygiene Laboratory in London. Addresses of the authorities to be contacted are furnished at the end of Part IV.

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